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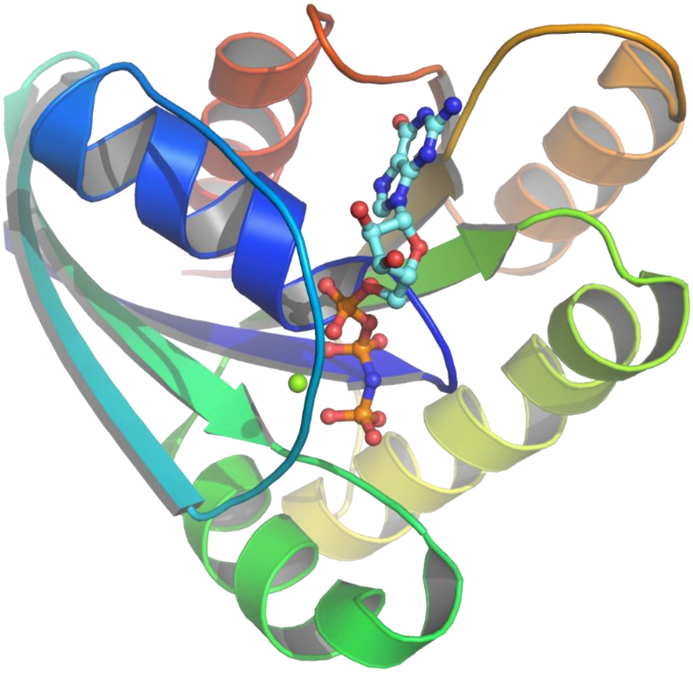

The Third
RAS INITIATIVE SYMPOSIUM
Abstract Book
May 24-26, 2021

Frederick National Laboratory
for Cancer Research

sponsored by the National Cancer Institute

U.S. Department of Health and Human Services | National Institutes of Health | National Cancer Institute

The Third RAS Initiative Symposium
Advanced Technology Research Facility, Frederick, MD
May 24 – 26, 2021

All times are shown in Eastern US Time

May 24

11:00 am – 11:10 am **Introduction** & **Welcome**
National Cancer Institute Director – Norman Sharpless
RAS Scientific Consultant – Frank McCormick

11:10 am – 11:40 am **Keynote Speaker:**
Charles Swanton
Francis Crick Institute
"Cancer Evolution, Immune Evasion and Metastasis"

11:40 am – 2:00 pm **RAS Biology (Session Chair: Frank McCormick)**

11:40am: Mariano Barbacid
CNIO - Spanish National Cancer Research Center
"Targeting KRAS Cancer: Target Inhibition vs Target Ablation"

12:10pm: Andy Aguirre
Dana-Farber Cancer Institute, Harvard Medical School
"Acquired Resistance to KRAS G12C Inhibition"

12:40pm: Pablo Rodriguez-Viciana
University College London
"The SHOC2 Phosphatase Complex: A Node for Selective Inhibition of the ERK Pathway in RAS-driven Cancers"

1:10pm: Marie Evangelista
Genentech
"CLAMPing Down on KRAS: Conformation Locking Antibodies to Drug KRAS-driven Tumors and to Investigate Drug Resistant Mechanisms"

1:40pm: **Short Talk:** Lauren Adams
Northwestern University
"Characterization of the KRAS Proteoform Landscape in Colorectal Cancer by Top-Down Proteomics"

2:00 pm – 3:00 pm **Break**

3:00 pm – 5:40 pm **Structure (Session Chair: Dwight Nissley)**

3:00pm: Dhirendra Simanshu
Frederick National Laboratory for Cancer Research
"Novel Structural Insights into RAS-effector Interactions"

3:30pm: Debbie Morrison
National Cancer Institute
"Structural Insights on How RAS Binding Mediates the B-RAF Monomer to Dimer Transition"

4:00pm: Michael Eck
Dana-Farber Cancer Institute, Harvard Medical School
"Insights into RAS/MAP Kinase Pathway Regulation and Pharmacology from cryo-EM Structures of BRAF/MEK/14-3-3 Complexes"

4:30pm: Chris Marshall
Princess Margaret Cancer Centre
"Calmodulin Regulates KRAS4b Localization Through Sequestration of the Farnesyl Moiety"

5:00pm: **Short Talk:** Jana Ognjenovic
Frederick National Laboratory for Cancer Research
"Cryo-EM Structure and Conformational Dynamics of Human Neurofibromin"

5:20pm: **Short Talk:** Timothy Wendorff
Genentech
"Oncogenic BRAF Mutations Overcome Negative Regulation of RAF Activity by ATP and Promote Dimerization to Activate MAPK Pathway Signaling"

May 25

11:00 am – 1:40 pm **RASopathies (Session Chair: Dom Esposito)**

11:00am: Kate Rauen
University of California, Davis
"Modeling RASopathies: Skeletal Myopathy in Costello Syndrome"

11:30am: Brigitte Widemann/Marielle Yohe
National Cancer Institute
Widemann*: "Neurofibromatosis type I"*
Yohe: *"Natural History Study of RASopathies"*

12:00pm: Maria Kontaridis
Beth Israel Deaconess Medical Center, Harvard Medical School
"Causal Mechanisms for RAF1-mediated Cardiac Hypertrophy in RASopathies"

12:30pm: Pau Castel
University of California, San Francisco
"Role of the RAS GTPase RIT1 in RASopathies and Cancer"

1:00pm: **Short Talk:** Antonio Cuevas Navarro
University of California, San Francisco
"Mutant RIT1 Compromises Mitotic Fidelity Through Suppression of the Spindle Assembly Checkpoint"

1:20pm: **Short Talk:** Russell Spencer-Smith
National Cancer Institute
"Germline RASopathy Mutations Provide Functional Insights into the RAF Cysteine-Rich Domain (CRD)"

1:40 pm – 3:00 pm **Break**

3:00 pm – 6:10 pm **Therapeutics (Session Chair: Caroline DeHart)**

3:00pm: David Turner
Frederick National Laboratory for Cancer Research
"Mapping the Surface of Mars: Identification of Novel Binding Pockets in KRAS"

3:30pm: Craig Crews
Yale University
"PROTAC-mediated K-RAS Degradation"

4:00pm: Rusty Lipford
Amgen
"Sotorasib: CodeBreaK 100 Registrational Phase 2 Results and Pre-clinical Update"

4:30pm: Kevan Shokat
University of California, San Francisco
"Identification of Drug Binding Pockets for Distinct Nucleotide States of Oncogenic K-RAS Mutants"

5:00pm: Karen Cichowski
Brigham and Women's Hospital, Harvard Medical School
"Developing Combinatorial Strategies for RAS-driven Cancers"

5:30pm: **Short Talk:** Xosé R. Bustelo
University of Salamanca
"RRAS2 Mutations Act as Fully Autonomous Cancer Drivers in a Variety of Tumor Types"

5:50pm: **Short Talk:** Luca Gerosa
Harvard Medical School
"Pulsatile Reactivation of MAPK Signaling Enables Persistence of Drug-adapted BRAF-mutant Melanoma Cells"

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May 26

11:00 am – 1:40 pm **Therapeutics II (Session Chair: Tommy Turbyville)**

11:00am: Mollie Leoni
Kura Oncology, Inc.
“Investigating Single Agent and Combination Tipifarnib Strategies to Treat HRAS Dependent Head and Neck Squamous Cell Carcinoma”

11:30am: Darryl McConnell
Boehringer Ingelheim
“Inhibitors and PROTACs for all KRAS Mutants”

12:00pm: Piro Lito
Memorial Sloan Kettering Cancer Center
“Regulators of the KRAS Nucleotide Cycle and Susceptibility to Emerging Therapeutics”

12:30pm: Bob Nichols
Revolution Medicines, Inc.
“RMC-6291: Biological Features of Targeting KRASG12C(ON) and Potential Application to Overcoming Drug Resistance in RAS-Addicted Tumor”

1:00pm: **Short Talk:** Oleksii Rukhlenko
University College Dublin
“Targeting Oncogenic RAS Using Next-Generation Modelling”

1:20pm: **Short Talk:** Matthias Drosten
CNIO - Spanish National Cancer Research Center
“KRAS4A Induces Metastatic Lung Adenocarcinomas in vivo in the Absence of the KRAS4B Isoform “

1:40 pm – 3:00 pm **Break**

3:00 – 5:30 pm **RAS Biology II (Session Chair: Andy Stephen)**

3:00pm: Dave Tuveson
Cold Spring Harbor Laboratory
“Models Identify New RAS Effectors in Pancreatic Cancer”

3:30pm: Channing Der
University of North Carolina
“Targeting KRAS Effector Signaling for the Treatment of Pancreatic Cancer”

4:00pm: Bill Hahn
Dana-Farber Cancer Institute, Harvard Medical School
"Systematic Approaches to Understand KRAS Function"

4:30pm: Arvin Dar
Icahn School of Medicine at Mount Sinai
"Structural Basis for the Action of Clinical MEK Inhibitors at KSR-MEK-RAF"

5:00pm: Christin Burd
The Ohio State University
"Unravelling the Mystery of NRAS Mutational Selectivity in Melanoma"

An asynchronous week-long poster session will be available to all registered participants for the full week: May 24th – May 28th.

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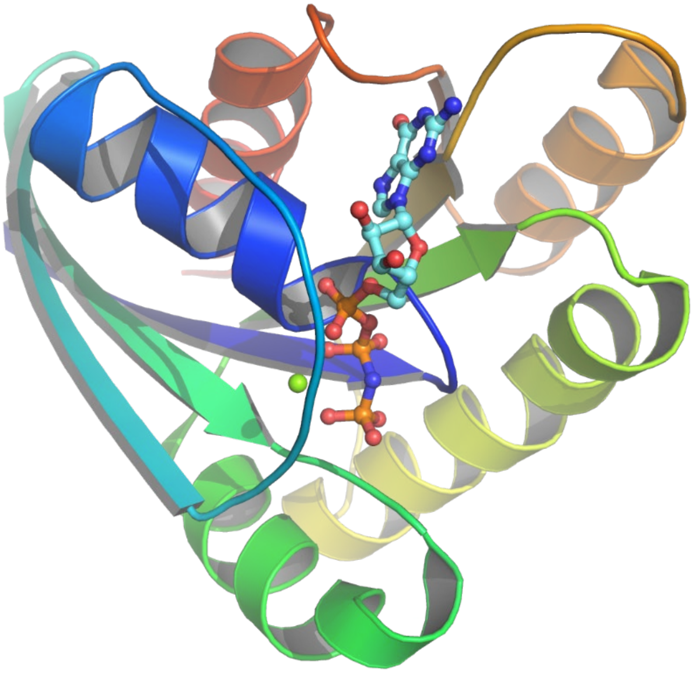

RAS Biology

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A EFR3A-PI4KA signaling axis regulates oncogenic KRAS signaling and tumorigenesis

Hema Adhikari[1], Walaa Kattan[2,3], Shivesh Kumar[4], Pei Zhou[4], John F. Hancock[2,3] and Christopher M. Counter[1]

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Oncogenic mutations in RAS genes (HRAS, NRAS and KRAS) are detected in a third of human cancers. To mediate oncogenic signaling, mutant RAS interacts with effectors through protein-protein interactions primarily at the plasma membrane (PM). Therefore, we sought to identify the protein networks engaged by each RAS isoform to reveal new vulnerabilities for future therapeutic development. To identify interactome components promoting RAS oncogenesis, we previously characterized the RAS interactome by BirA-mediated proximity labeling, and then through CRISPR/Cas9 loss-of-function genetic screen, determined the interactome components that mediate RAS oncogenesis. Mining these datasets identified EFR3A as a strong candidate. EFR3A is an adapter protein that recruits the druggable phosphatidylinositol kinase (PI4KA) to the PM to generate the PI4P. We confirmed that EFR3A is indeed a component of the RAS interactome and preferentially interacts with the oncogenic form of RAS when expressed recombinantly or in cells. Genetic ablation of EFR3A reduced KRAS effector signaling, transformation, and tumorigenic growth. Epistatic analysis as well as electron microscopy revealed that EFR3A functions at the level of RAS itself, fostering the occupancy and nanoclustering of the oncoprotein at the PM to promote signaling and transformation. Finally, we show the clinical potential of this signaling axis by demonstrating that PI4KA inhibitor synergizes with emerging $KRAS^{G12C}$ inhibitors. In conclusion, we identify EFR3A as a potential effector of oncogenic KRAS that forms a feedback-like circuit that enhances oncogenic RAS signaling, thereby providing a pharmacologic target to enhance the anti-neoplastic activity of drugs targeting oncogenic RAS or its effectors.

B-cell chronic lymphocytic leukemia is driven by overexpression of the unmutated RAS-related 2 gene

Alejandro M. Hortal[1], Clara L. Oeste[1], Claudia Cifuentes[1], Miguel Sánchez Alcoceba[2], María Isabel Fernández-Pisonero[3], Rut Tercero[1], Pilar Mendoza[1], Verónica Domínguez[4], Belén Pintado[4], David Abia[1], Carmen García-Macías[3], Xosé R. Bustelo[3], Marcos González Díaz[2], & Balbino Alarcón[1]

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SUMMARY

Among the most frequently mutated oncogenes are the classic RAS members which are found with activating, oncogenic, mutations in approximately 13% of all human cancers. However, the large majority of human cancers, including B-cell chronic lymphocytic leukemia (B-CLL), do not bear such mutations. Here we show that mice overexpressing the wild type form of RAS-related-2 develop a progressive indolent leukemia that resembles human B-CLL. This suggested that overexpression of unmutated RRAS2 could drive B-CLL in humans. Indeed, our data show that wild type RRAS2 mRNA is overexpressed in 96% of human B-CLL. In addition, RRAS2 expression is higher in patients with full blown CLL than in those with a premalignant monoclonal B cell lymphocytosis (MBL). Most interesting, we have found that a single nucleotide polymorphism (rs8570) in the 3’ UTR of the RRAS2 mRNA is associated to higher expression, more incidence of CLL versus MBL and to leukemias harboring unmutated Ig heavy chain (IgH) in male patients. IgH mutational status and male gender are risk factors associated with poorer prognosis. Our results strongly point out to RRAS2 overexpression as causative of B-CLL and questions the requirement of oncogenic mutations in RAS family members to induce cancer.

Prevalence of *KRAS* and concomitant mutations in advanced lung adenocarcinoma patients

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[2]Departamento de Tecnologias da Informação e Educação em Saúde (DTIES) da Faculdade de Ciências Médicas (FCM) na Universidade do Estado do Rio de Janeiro (UERJ), Rio de Janeiro, Brazil
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Background: One of the most frequently mutated oncogenes in cancer belongs to the Ras family of proto-oncogenes, which encode distinct key signalling events. RAS gain-of-function mutations are present in ~30% of all human cancers, where *KRAS* is the most frequent mutated isoform being altered in different cancer types including lung cancer.

Objectives: To analyse, retrospectively, the incidence of *KRAS* mutations, with an emphasis in the evaluation of G12C mutations, and also to investigate the presence of *KRAS* concomitant mutations.

Methods: In this retrospective study, we analysed genomic DNA extracted from paraffin embedded tumour tissues from 121 Brazilian advanced lung adenocarcinoma patients in order to evaluate via Next Generation Sequencing (NGS) the incidence of *KRAS* mutations and correlate, when possible, to clinicopathological characteristics such as sex. The prevalence of co-occurring mutations alongside *KRAS* was also investigated. Statistical analysis was performed to investigate the association between mutation status, mutation types and sex.

Results: There was a prevalence of male (N= 63; 54.8%) in comparison to female (N= 52, 45.2%) patients in our cohort. *KRAS* was mutated in 20.86% (24/115) of all samples. Of the total number of *KRAS* mutants (N=24), G12D (N= 6; 24%) was the most frequent mutation type, whereas G12C came in third place alongside G12A (N=3; 12,5%). Interestingly, 33,3% of the mutant *KRAS* samples showed other co-occurring mutations. There was no significant association with sex.

Conclusion: This study confirms the prevalence of the most frequent *KRAS* mutations in advanced lung cancer and reveals the presence of rare concomitant mutations alongside *KRAS*. Further investigation on the importance of these concurrent genomic alterations in patient prognosis and treatment response is warranted.

Keywords: *KRAS*, advanced lung adenocarcinoma, concomitant mutations, NGS

New tools to study the role of RAS/CRAF interaction in RAS-driven lung cancer.

Romain Baer, Chris Moore, Ian Rosewell, Nourdine Bah, Miriam Molina-Arcas, David Hancock and Julian Downward

The RAF/MAPK pathway is a major RAS effector pathway implicated in RAS oncogenic properties, regulating a diversity of cellular processes. As RAF kinases are thought to be attractive therapeutic targets in RAS-driven cancers, preclinical studies focusing on targeting only the CRAF kinase revealed effective inhibition of tumour development without inducing significant systemic toxicities, in a model of KRAS-driven lung tumour initiation. Recently, Sanclemente *et al.* further demonstrated that complete ablation of CRAF in established advanced lung tumours ($\textit{Kras}^{G12V}$/*Trp53*) triggers sustainable tumour regression, despite CRAF kinase activity being dispensable. This approach validates CRAF as a powerful therapeutic target for KRAS-driven lung cancer.

RAS-GTP strongly interacts with CRAF through its Ras Binding domain (RBD). This interaction is required to activate CRAF. Surprisingly, disruption of this interaction has never been proven to be a therapeutic target *in vivo*. Using CRISPR/Cas9 technology, we engineered a new mouse model in which CRAF RBD is constitutively mutated to completely abolish its interaction with RAS GTPases ($\text{CRAF}^{\text{R89L}}$). This mouse model recapitulates the embryonic lethal phenotype of the constitutive CRAF KO mouse. We engineered two murine lung cancer cell lines ($\textit{Kras}^{G12V}$/*Trp53*) that are heterozygous for this mutation ($\text{CRAF}^{\text{R89L/KO}}$). Surprisingly, CRAF ablation or RBD disruption minimally affects ERK signalling regardless of an enrichment in GTP-bound RAS. However, our mutant cell lines display a PI3K/AKT positive feedback. CRAF ablation or RBD disruption drastically reduces tumour growth *in vivo* in a syngeneic transplant model. Our work highlights CRAF interaction with RAS as a new therapeutic target for KRAS-driven lung cancer.

Tollip-dependent perinuclear localization of CK2 signaling endosomes is an essential feature of KRAS mutant tumors

Srikanta Basu[1], Sandip K. Basu[1], Ave Keefer[1], Nancy Martin[1], Karen Saylor[1], Brian T. Luke[2], Sudipto Das[3], Thorkell Andresson[3], Norene O'Sullivan[4], Serguei Kozlov[4] and Peter F. Johnson[1]

Authors' Affiliations: [1]Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute, Frederick, Maryland; [2]Advanced Biomedical Computational Science, Frederick National Laboratory for Cancer Research, Frederick, MD, USA; [3]Protein Characterization Laboratory, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research, Leidos Biomedical Research, Inc., Frederick, MD, USA; [4]Center for Advanced Preclinical Research, National Cancer Institute, Frederick, Maryland

Our laboratory has shown that oncogenic RAS induces perinuclear translocation of the RAS effector kinases, p-ERK and CK2, along with the signaling scaffold KSR1, forming signaling hubs termed "perinuclear signaling centers" or PSCs. PSCs can also be induced transiently by serum stimulation of normal cells, appearing with delayed kinetics (4-6 h post-stimulation). PSCs are present in cancer cell lines and tumor tissues but not in normal cells, suggesting that subcellular compartmentalization of oncogenic kinases drives tumorigenesis. PSCs occur on perinuclear endosomes, where KSR1 and CK2, but not ERK, are associated with recycling (Rab11A positive) endosomes. We show here that the ubiquitin adaptor protein, Tollip, is required for perinuclear localization of Rab11A$^+$ endosomes containing CK2 and KSR1. This involves the ability of Tollip to tether endosomes to the endoplasmic reticulum (ER). Tollip physically interacts with KSR1 through the KSR1 pseudo-kinase domain and a highly conserved "linker" region in Tollip. Accordingly, Tollip colocalizes with CK2, KSR1 and Rab11A, but not ERK, on ER-associated perinuclear endosomes. Notably, KRAS mutant tumor cells are dependent on Tollip for proliferation/survival, whereas HRAS and NRAS-transformed cells and non-transformed cells are relatively unaffected by Tollip silencing. Furth*ermore, KRas*^{G12D}-driven lung tumors in Tollip KO mice did not advance beyond the adenoma stage, showing that Tollip is required for progression to malignancy. Phospho-proteomic analysis of serum-stimulated cells revealed that perinuclear CK2 selectively phosphorylates proteins involved in ribosome biogenesis and eukaryotic translation initiation, likely contributing to the oncogenic function of PSCs. One such substrate, the atypical kinase RIOK1, was phosphorylated on Ser21/22, a putative CK2 site. RIOK1 is essential for 18S ribosomal RNA maturation and a known synthetic lethal gene for KRAS mutant tumors. Preliminary results show that Ser 21/22 phosphorylation is stimulated by KRAS^{G12V}, indicating that perinuclear CK2 drives RIOK1 phosphorylation. Overall, our findings identify Tollip as a novel effector of KRAS-driven tumorigenesis that may represent a specific, targetable vulnerability of these cancers.

All KRAS mutants are equal but some are more equal than others

Suzan Ber[1], Khushali Patel[1], Annie Howitt[1], Pablo Oriol Valls[1], Alessandro Esposito[1]

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The notion that specific oncogenic KRAS alleles trigger distinct biological responses that promote carcinogenesis only in permissive tissues is rapidly gaining momentum. Yet the mechanisms underpinning the pathophysiology of specific KRAS mutations are not well understood limiting our capabilities for disease management.

KRAS is a signalling hub at the intersection between cell signalling and metabolism. We attempted to establish causal links between specific genetic alterations, signalling dynamics and cellular phenotypes. For this reason, over the last years we worked with isogenic colorectal cancer cell lines (SW48 and LIM1215) harbouring heterozygous mutations at codon 12 (G12A, G12C, G12D and G12V) as well as inducible cell lines and reporter cell lines for signalling (*e.g.*, ERK activity) and metabolic (*e.g.* ATP concentration) pathways.

Here we will examine the distinct signalling and metabolic signatures that we reveal through gene expression analysis and metabolomics. We observe alterations in glutamine metabolism in all KRAS-mutant cells. However, we reveal also significant differences across oncogenic alleles in glutamine synthesis and nitrogen recycling pathways mediated, for example, by the differential expression of GLUL. In limited nutrient conditions, the altered balance between glutamine catabolism and synthesis results into drastically different mutant-specific fitness. We attribute these differences to mutant-specific alternations in FOXO1 and mTOR signalling.

Taken together, our observations indicate a possible mechanism underpinning differences between KRAS alleles that might mediate differential fitness in different contexts. In the future, we will attempt to integrate data at multiple scales from single-cell dynamics to system approaches aiming to dissect the contribution of both genetic and non-genetic heterogeneity in KRAS-driven carcinogenesis.

Uncovering mechanisms of immune evasion in a novel immunogenic model of KRAS-mutant lung cancer

Jesse Boumelha, Sophie de Carné, Pablo Romero, Edurne Mugarza, Miriam Molina & Julian Downward

The Francis Crick Institute, London UK

Oncogenic KRAS mutations drive tumourigenesis in 30% of non-small cell lung cancer (NSCLC). Despite much effort, targeted therapies that aim to directly inhibit signalling pathways downstream of KRAS have limited clinical benefits for NSCLC patients, but the recent approval of PD-1/PD-L1 antibodies has led to striking durable responses. However, only a fraction of patients respond and therefore a deeper understanding of the mechanisms that drive immune evasion are required in order to broaden the clinical efficacy of immunotherapy. Increasing evidence suggests that oncogenic signalling pathways greatly influence the tumour immune landscape to impair anti-tumour immune responses. We therefore aim to understand the mechanisms by which KRAS signalling mediates immune evasion in lung cancer. Current mouse models of KRAS-mutant lung cancer are poorly immunogenic, limiting investigations into tumour-immune interactions. To overcome this, we generated a novel transplantable KRAS-mutant lung cancer model, KPAR1.3, which triggers spontaneous anti-tumour immune responses and is sensitive to immune checkpoint blockade. To identify mechanisms of immune evasion we carried out an *in vivo* pooled CRISPR-Cas9 screen targeting 240 KRAS-regulated genes using this novel immunogenic model. This identified a number of genes that increased sensitivity or caused resistance to anti-tumour immune responses. As an alternative approach we utilised the recently developed class of mutant-specific KRAS-G12C inhibitors to assess the impact of inhibiting KRAS signalling on anti-tumour immune responses in KPAR1.3 tumours. KRAS-inhibition stimulated adaptive immunity *in vivo*, which contributed to the response of KPAR1.3 tumours in immune-competent mice. Together these data suggest that targeting KRAS, or KRAS-driven mechanisms of immune evasion, could broaden the clinical efficacy of immunotherapy in KRAS-mutant NSCLC.

Mutant Kras G12V upregulates cholesterol and lipid biosynthesis in mouse colon epithelial cells

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[2]Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19104, USA
[3]Biostatistics and Bioinformatics Facility, Fox Chase Cancer Center, Philadelphia, PA, USA

Oncogenic *KRAS* mutations are prevalent in colorectal cancer (CRC) and are associated with poor prognosis and resistance to therapy. There is a substantial diversity of *KRAS* mutant alleles observed in CRC. Importantly, emerging clinical and experimental analysis of relatively common *KRAS* mutations affecting amino acids G12, G13, A146, and Q61 suggest that each mutation differently influences the clinical properties of a disease and response to therapy. Although there is emerging evidence to suggest biological differences between mutant *KRAS* alleles, these are yet to be fully elucidated. Further exploration of allele-specific differences may provide evidence for allele-specific therapeutic potential. One approach to study allelic variation involves the use of isogenic cell lines that express different endogenous *Kras* mutants. Here, we generated *Kras* G12D and G12V isogenic $Apc^{-/-}$ mouse colon epithelial cell lines to study functional differences between *Kras* mutations. The cell lines were generated using CRISPR-driven genome editing by altering the original G12D *Kras* allele to G12V. We utilized these cell lines to perform transcriptomic and proteomic analysis to compare different signaling properties between the two mutants. Both screens indicate significant differences between G12D and G12V *Kras* mutants in pathways relating to cholesterol and lipid regulation. We found that these processes are upregulated in G12V lines through increased expression of nuclear SREBP1 and higher activation of mTOR signaling compared to G12D lines. Consistently, G12D lines were more sensitive to lipid depletion and to the combination of lipid depletion and MEK inhibition than G12V lines. These observations highlight differences between *Kras* G12D and G12V mutant cell lines in their signaling properties. Further exploration of these pathway may prove to be valuable for understanding how specific KRAS mutants function, and identification of novel, mutant-specific therapeutic opportunities in CRC.

The Glucocorticoid Receptor Interacts with RAS Proteins with implications in lung cancer

Bozhena Caratti[1], Miray Fidan[1], Giorgio Caratti[1], Kristina Breitenecker[2], Rainer Wittig[3], Rebecca Traut[1], Emilio Casanova[3], Jan Tuckermann[1§#], Herwig Moll[3§#], Ion Cirstea[1§#]

[1]Institute of Comparative Molecular Endocrinology, University of Ulm, Ulm, Germany; [2]Department of Physiology, Center of Physiology and Pharmacology, Comprehensive Cancer Center (CCC), Medical University of Vienna, Vienna, Austria; [3]Institute for Laser Technologies in Medicine & Metrology (ILM) at Ulm University, Ulm, Germany; [#]Equal contribution; [§]Corresponding author

Activating RAS mutations are involved in a wide variety of cancers. The glucocorticoid receptor (GR), a member of the nuclear receptor family, has been proposed to interact with components of the MAPK and AKT pathways downstream of RAS, inhibiting their activation. We show that GR interacts with RAS in the absence of ligand and inhibits activation of mutant KRAS shown in lung cancer A549 cells. Addition of ligand releases GR-mediated inhibition of RAS and AKT, MEK1/2 and p38 signaling. CRISPR-Cas9- mediated deletion of GR in A549 cells enhances tumor growth in xenografts in mice. In patient samples of non-small cell lung carcinomas GR expression is diminished accordingly and lower GR expression correlates with a worse disease outcome. Thus, the interaction of unliganded GR with RAS is a novel non-canonical signaling mechanism regulating tumor growth.

Lipid-Dependent Dynamics of RAS Revealed using Multiscale Machine-Learned Modeling Infrastructure (MuMMI)

Timothy S. Carpenter on behalf of the JDACS4C Pilot 2 team.

Investigating the detailed mechanism of RAS signaling complex initiation requires a combination of both near-atomic detail and also macroscopic temporal and spatial scales. This combination of scope and resolution is not possible with conventional computational or experimental techniques.

We demonstrate here the multiscale machine-learned modeling infrastructure (MuMMI) that uses machine learning to create a scale-bridging ensemble of greater than 100,000 simulations of active wild-type KRAS on a complex, asymmetric membrane. MuMMI enables the coupling of a macro-scale model at experimentally relevant temporal and spatial scales with a vast ensemble of higher resolution coarse-grained micro-scale simulations.

Through this extensive sampling over a large range of system conditions we are able to identify distinctive patterns of local lipid composition that correlate with RAS multimerization. The different compositions, or 'lipid fingerprints', are coupled to various aspects of RAS dynamics. The lipid environments are also predicted to influence effector binding, and thus could potentially act as a mechanism for regulating cell signaling cascades.

Further expansion of MuMMI has recently been achieved by incorporating a third (all-atom) resolution scale, multilevel feedback, higher fidelity macro model and support for different protein types. We are currently running a large-scale simulation campaign of RAS and RAF proteins on a complex plasma membrane.

KRAS conveys anti- and pro-invasive signals in response to fibroblast-secreted factors

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[5]INEB – Institute of Biomedical Engineering, University of Porto, Porto, Portugal

Oncogenic KRAS influences not only autocrine mechanisms, but also the communication with the tumor microenvironment (TME). Given the relevance of this oncogene in prognosis and therapy as well as the inexistence of a targeted therapy for mutant KRAS patients, there is an urgent need to better understand KRAS-associated signals. The TME plays important roles in cancer aggressiveness and cancer-associated fibroblasts, recognized as major stromal components, are known drivers of colorectal cancer (CRC) invasion. Herein, we aimed to explore and characterize the role of mutant KRAS in mediating CRC cells-fibroblast crosstalk.

Our results revealed that CRC cell lines display different invasive responses (responsive/non-responsive) to fibroblasts conditioned media. Also, KRAS silencing results in opposite effects: increasing or decreasing the invasive potential. A phospho-RTK array using two representative cell lines revealed the HGF/C-MET axis as a modulator of fibroblasts-induced invasion in responsive cells. Neutralization/supplementation experiments showed that HGF-induced invasion was KRAS-dependent. The relevance of C-MET-HGF axis in the context of mutant KRAS CRC liver metastasis is being assessed in an animal model. The array also pinpointed HER3 as a possible modulator underlying the pro-invasive properties upon KRAS inhibition in the non-responsive cell line. However, HER3 inhibition did not suppress KRAS-silencing induced invasion. The putative mechanism underlying this effect is under study.

This work awards KRAS a role in the response to fibroblast-secreted factors that induce invasion. Nevertheless, the opposite effects observed upon KRAS silencing challenge the classic KRAS oncogenic role, highlighting the need to carefully stratify patients that may benefit from anti-KRAS therapies.

Exploring the role of RAS signalling in the remodelling of the tumour microenvironment of KRAS-driven lung cancer

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Lung cancer is the leading cause of cancer-related death with a survival rate of less than 5%, mostly due to patients presenting with metastatic disease and developing resistance to therapy. Recently, molecularly-targeted agents (e.g. against EGFR or ALK) and immunotherapies (anti-PD1 antibodies) have been approved for treatment of NSCLC. However, it remains a challenging disease, particularly in KRAS-mutated cases, which are associated with an even worse prognosis and do not benefit from targeted agents. KRAS inhibitors currently in clinical development hold promise, but only for patients with a specific, relatively uncommon, mutation (G12C).

We have found that RAS signalling through PI3K has a significant impact on tumour progression by acting over the tumour microenvironment. Proteomic analysis of KRAS-driven lung tumours lacking RAS-PI3K interaction suggested a dependency for this signalling pathway in functions typically related to CAFs. Further analysis confirmed that TGF-□ activated fibroblasts deficient for RAS-PI3K interaction displayed changes in CAFs markers such as YAP1, □-SMA, vimentin or fibronectin. This was accompanied by a reduction in the ability to contract collagen gels, impairment in cytoskeleton rearrangement and formation of thinner and more disorganised matrices. Furthermore, our data also revealed that proliferation of KRAS mutant lung tumour cells is highly impaired in those matrices generated by CAFs lacking RAS-PI3K. *In vivo* analysis of tumour stiffness showed that lung tumours lacking RAS-PI3K interaction are softer.

In summary, our data suggest an overarching effect of RAS signalling through PI3K in the formation of a pro-tumorigenic extracellular matrix by controlling CAF activation and function to modulate tumour cell behaviour. The high prevalence of RAS mutations in human cancer and the presence of CAFs in all tumours means that these results have far-reaching implications and point to new ways to tackle RAS-driven tumours.

Predicted ‘wiring landscape’ of Ras network in 29 human tissues

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Ras is an important hub protein at the head of numerous signaling pathways and plays a starring role in various types of cancers, notably in pancreas, colon and lung adenomas. The usual suspects are three oncogenic isoforms - i.e. HRAS, KRAS and NRAS - that are highly mutated and drive tumorigenesis [1]. Our study is based on the paradigm of network medicine that sees disease as a perturbation of a network of interconnected proteins finely orchestrating cell's physiology and phenotype through the onset of downstream signal transduction. As such, we built a mechanistic model of the interactions of the three Ras oncoproteins with their direct interactors (known as "effectors"), with protein abundances and binding affinities being the system's parameters, in order to study elementary pathological and physiological conditions of Ras network [2].

Using high-quality proteomic data from 29 (healthy) human tissues [3], we quantified the amount of individual Ras-effector complexes, and characterized the (stationary, reference) Ras “wiring landscape” specific to each tissue. We simulated mutant- and stimulus-induced network re-configurations, miming respectively cancerous and physiological state, and compared them to the reference network.

Moreover, we investigated the contribution of the input parameters (binding affinities and effector concentrations) in determining the complex formations underlying the specific wiring landscape, by 3D data interpolation onto (tissue-specific) surfaces. This revealed that high affinity - more than high concentration, - is critical for complex formation. As a consequence, we analyzed local and global binding affinity fluctuations and assessed their impact on the system's robustness [4]. Further research will aim at the calibration of the binding affinity parameters, based on the Ras-effector complexes and the activation of the associated downstream pathway.

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KRAS-Dependent Cancer Cells Promote Survival by Producing Exosomes Enriched in Survivin

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Mutations in *KRAS* frequently occur in human cancer and are especially prevalent in pancreatic ductal adenocarcinoma (PDAC), where they have been shown to promote aggressive phenotypes. However, targeting this onco-protein has proven to be challenging, highlighting the need to further identify the various mechanisms used by KRAS to drive cancer progression. Here, we considered the role played by exosomes, a specific class of extracellular vesicles (EVs) derived from the endocytic cellular trafficking machinery, in mediating the ability of KRAS to promote cell survival. We found that exosomes isolated from the serum of PDAC patients, as well as from KRAS-transformed fibroblasts and pancreatic cancer cells, were all highly enriched in the cell survival protein Survivin. Exosomes containing Survivin, upon engaging serum-starved cells, strongly enhanced their survival. Moreover, they significantly compromised the effectiveness of a novel therapy that combines an ERK inhibitor with chloroquine, which is currently in clinical trials for PDAC. The survival benefits provided by oncogenic KRAS-derived exosomes were markedly reduced when depleted of Survivin using siRNA or upon treatment with the Survivin inhibitor YM155. Taken together, these findings demonstrate how *KRAS* mutations give rise to exosomes that provide a unique form of intercellular communication to promote cancer cell survival and therapy resistance, as well as raise interesting possibilities regarding their potential for serving as therapeutic targets and diagnostic markers for KRAS-dependent cancers.

Oncogenic RAS activity increases interstitial adenosine to promote immune evasion in lung adenocarcinoma

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KRAS mutations occur in 32% of lung adenocarcinomas (LUAD), leading to more aggressive disease and resistance to therapy in preclinical studies. However, the association between *KRAS* mutational status and patient outcome or response to treatment remains unclear, presumably due to additional events modulating RAS pathways.

To obtain a broader measure of RAS pathway activation, we developed RAS84: a transcriptional signature optimised to capture oncogenic RAS activity in LUAD. We report that 84% of LUAD show clear evidence of RAS oncogenic activation, falling into four groups characterised by coincident mutation of *STK11/*LKB1, *TP53* or *CDKN2A*. In two independent LUAD cohorts, we show that RAS84 stratification predicts outcome and response to chemotherapy, whereas the KRAS mutation status does not. Despite leading to poor outcome, tumours with the highest oncogenic RAS activity level are infiltrated with leucocytes and express IFN-stimulated genes (ISGs), necessary for a potent anti-tumoural immune response. They also express high levels of several immune checkpoint genes (*e.g., CD274* (PD-L1), *PDCDL2* (PD-L2), *PVR*, *TIGIT*) and deregulate genes related to adenosine metabolism or function (*e.g., NT5E* (CD73), *ADORA2B, SLC29A4* (ENT4)), suggesting immune inhibition. Using MEK or KRASG12C inhibitors, we validated the RAS-MAPK-dependent regulation of adenosine genes *in vitro* and mouse models and the direct regulation of interstitial adenosine.

Our data demonstrate that oncogenic RAS activity induces immune evasion by increasing interstitial adenosine and suggest that RAS transcriptional activity is more informative than *KRAS* mutation status to study oncogenic RAS in large cohorts of patients and could eventually help clinical decision.

RRAS2 activating mutants promote ovarian tumor formation

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RRAS2 gain-of-function (GOF) mutations have been identified at low frequency in recent PanCancer studies. However, the cancer driver and pathobiological roles of this GTPase remain poorly characterized *in vivo*. To tackle those issues, we have followed a two-pronged approach. Firstly, we used a *Cre*-regulated knock-in model to express a GOF R-Ras2 mutant (Q72L) either systemically or in an ovary-specific manner in mice. Using this method, we have found that the expression of this GOF mutant triggers follicular atresia-induced infertility and, subsequently, ovarian cystadenomas at high penetrance. This is associated with the dedifferentiation of a subset of ovarian cells back to a male sex determination developmental state. These tumors can be blocked by PI3K and mTORC inhibitors. By contrast, they are MEK- and RalGDS-independent. Interestingly, we have found human tumors with the same biomarkers in clinical samples. Secondly, we used gene-editing techniques to eliminate endogenous wild-type or the *RRAS2*^{Q72L} mutant versions of *RRAS* in specific ovarian cancer cell lines. Elimination of endogenous R-RAS2^{Q72L}, but not of the wild-type counterpart, impairs the tumorigenic potential of these cells both in culture and xenotransplants. This is connected with reduced levels of MEK-ERK and PI3K-AKT signaling, polysomal translation rates, and basic metabolic activities. These defects emerge despite the presence of concurrent BRAF and *PI3KCA* GOF mutations in the cancer cells. These data indicate that *RRAS2*^{Q72L} mutations do play cancer driver roles in ovarian tumors. They are also consistent with R-RAS2 playing non-redundant roles with classical RAS oncoproteins in this process.

ERK dimerization promotes migration and invasion

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Introduction

Activation of the Ras-ERK signaling cascade is associated with increased metastasis risk in breast cancer. However, the mechanisms of ERK promoting tumorigenesis in breast cancer remain unclear. In the present study, we demonstrated that ERK dimers enhanced the invasion and migration of breast tumors cells.

Material and method

ERK dimers were detected by western blot in native-PAGE after using ERK dimer inhibitor and MEK inhibitor in breast cell lines, MCF-7 and MDA-231-MB. ERK dimers downstream targets were determined by mass spectrometry and confirmed by co-immunoprecipitation assays. The role of ERK dimers, KSR1, and actin filaments cytoskeleton in migration and invasion of breast cancer cells were analyzed by transwell and 3D-spheroid assay. KSR1-ERK dimers and actin colocalization were measured by immunofluorescence.

Results and discussion

ERK regulation is dependent on its dimerization in response to stimulation. The worst prognosis is associated with ERK dimerization upon EGF in comparison with ERK monomers upon IGF-1 stimulation in the MCF-7 cell line. We found that ERK dimerization upon EGF stimulation increased migration and invasion of breast tumor cells. Moreover, KSR1 overexpression induced ERK dimerization in the cytoplasm enhancing migration and invasion. In contrast, ERK dimers inhibition blocked invasion of MDA-231-MB cells.

Conclusion

ERK dimerization plays a crucial role in the migration and invasion of breast tumor cells. ERK dimers bind to the scaffold protein KSR1 to enhance migration. Blocking ERK dimers formation reduced invasion capacity of MCF-7 and MDA-231-MB tumors cells. Thus, targeting ERK dimerization could emerge as a valid therapeutic strategy for the treatment of breast cancer.

Towards identifying small molecules to chemically rescue deactivation of mutant KRAS using a novel computer simulation

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Cancer affects millions globally. Approximately 30 percent of cancers are driven by mutations in RAS proteins, which play a critical role in signaling cascades that regulate cell growth and proliferation. Many oncogenic Ras mutations prevent GTPase activating proteins (GAPs) from catalyzing the hydrolysis of GTP and subsequent deactivation of RAS, leading to uncontrolled cell growth. Two mutations of interest, G12V and G13D, prevent this catalysis by blocking the entry of the catalytic arginine finger on GAP. Here, we present our work towards identifying small molecules that can chemically rescue deactivation of mutant KRAS using a novel computer simulation. Our approach uses molecular dynamics to drive conformational changes, while periodically using a coupled Monte Carlo simulation to propose changes to the drug candidate. Application to wild type, G12V, and G13D KRAS with increasingly large libraries of molecular candidates allowed us to improve our method and generate collections of small molecules that may bind to Ras-GTP at high concentrations.

PIK3C3 is an Obligate Haplo-insufficient Tumor Suppressor Gene in *KRAS*-driven Lung Adenocarcinoma

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Sequencing of lung adenocarcinoma (LUAD) revealed frequent (~47%) heterozygous loss or rare mutations of *PIK3C3*, which encodes the Class III PI3K hVPS34. hVPS34 forms complexes with hVPS15, BECLIN1, and other proteins to regulate autophagy, endocytosis, and mTOR, but its role in LUAD has not been explored. *In vitro* studies showed that hVPS34 mutants preferentially impaired BECLIN1/UVRAG-containing complexes, diminished autophagy, and cooperated with mutant *RAS* in transformation assays. To assay effects on tumor formation, we delivered Ad5-SPC-Cre intratracheally to LSL-*Kras*$^{G12D/+}$;*Pik3c3*$^{f/+}$ and LSL-*Kras*$^{G12D/+}$;*Pik3c3*$^{f/f}$ mice. Compared with LSL-*Kras*$^{G12D/+}$ mice, LSL-*Kras*$^{G12D/+}$;*Pik3c3*$^{f/+}$ mice developed more and faster growing tumor nodules and had shorter survival. *Kras*-mutant LUAD cells with *Pik3c3* heterozygous deletion also proliferated faster in 3D cultures. Cells from *Kras*$^{G12D/+}$;*Pik3c3*$^{f/+}$ tumors also proliferated faster in 3D culture. Similar results were obtained with compound mutants of *Pik3c3*$^{f/+}$ and *KRAS*$^{G12C/+}$ but not *Braf*$^{V600E/+}$. Consistent with the *in vitro* assays, heterozygous *Atg5* deletion also accelerated tumorigenesis in *Kras*$^{G12C/+}$ mice. By contrast, homozygous *Pik3c3* deletion restored tumor incidence and rate to that on parental *Kras*-mutant mice. Tissue-Mass Spectrometry identified 194 upregulated and 262 downregulated proteins in *Kras*$^{G12D/+}$;*Pik3c3*$^{f/+}$ tumor nodules. Upregulated proteins were highly enriched for immune-related and Wnt signaling pathways. TCGA data showed that patients with *KRAS* mutant/*Pik3c3*$^{+/-}$ LUAD had higher *WNT5A* and *WNT5B* expression. Our results indicate that *PIK3C3* is an obligate haplo-insufficient tumor suppressor gene in *KRAS*-mutant LUAD and suggest that incomplete autophagy inhibition might have deleterious therapeutic effects. By contrast, *PIK3C3* heterozygosity might be a biomarker for tumors that would be sensitized to autophagy inhibition.

YAP1 transcriptional activity modulates KRAS^{G12C}-mutant cancer responses to KRAS^{G12C} inhibition

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Activated mutants of *KRAS* comprise the major oncogenic drivers in lung (LAC), colorectal (CRC), and pancreatic ductal adenocarcinoma (PDAC). Once considered “undruggable”, KRAS mutants harboring a G12C substitution (KRAS^{G12C}) can now be directly targeted pharmacologically. Excitingly, early-stage clinical observations in LAC indicate potent KRAS^{G12C}-inhibitor (G12Ci) anti-tumor activity, with an overall objective response rate (ORR) of ~40%. That said, reported response rates are significantly lower for CRC and PDAC. In addition to *de novo* resistance, a limitation for essentially all targeted therapies is treatment-induced acquired resistance in initially sensitive tumors, as reported recently for G12Ci (i.e., in LAC patients). One potential, yet currently undescribed, mechanism of resistance to G12Ci is activation of the transcriptional co-activator paralogs, YAP1 and TAZ. Following genetic silencing of *KRAS*, YAP1 activation has been described to overcome cancer cell addiction to mutant KRAS in LAC, CRC, and PDAC. To address whether YAP1 activation can similarly bypass sensitivity to G12Ci, we ectopically overexpressed doxycycline-inducible, constitutively active YAP1 (YAP1^{S127A}) in a panel of KRAS^{G12C}-mutant LAC, CRC, and PDAC cell lines. In all cell lines tested, overexpression of YAP1^{S127A} substantially diminished the cytostatic and cytotoxic effects of the G12Ci, MRTX1257, a structural analog of the clinical stage G12Ci, MRTX849/adagrasib (Mirati Therapeutics). Likewise, in the same cell lines, concurrent knockdown of YAP1/TAZ demonstrated a 4-to-28-fold enhancement of G12Ci efficacy in cell viability assays. Ongoing studies will determine whether pharmacologic inhibition of YAP1/TAZ transcriptional activity represents a therapeutically tractable (and novel) means by which to synergistically enhance G12Ci sensitivity.

RAS oncogenic activity defines unique oncogenic fates

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RAS family oncogenes demonstrate a tropism of specific driver mutations towards different cancers. Understanding the impact of individual RAS mutations on oncogenic activity, signaling, and tissue-specific cellular responses could inform on the origin of cancer, and development of potential therapies. To genetically interrogate how each mutation could differentially alter RAS signaling output *in vivo*, we created a panel of mice whereby two very different oncogenic mutations, G12D, and Q61R, were expressed from an inducible *Kras* allele encoded with native or common codons to generate low or high levels of oncoprotein. These four genetically engineered mice were then crossed into a lung-specific *CC10-CreER* background, demonstrating that the tumorigenicity of these alleles tracked with oncogenic activity: *comQ61R* > *comG12D* > *natQ61R* > *natG12D*. To further evaluate the impact of these alleles on cancer tropism, we crossed these alleles into a *Rosa26-CreERT2* background. Activating these alleles globally induced a restricted number of tumors, with increasing active Kras leading to increased disease severity and decreased survival. Histopathological analysis revealed highly aggressive myeloproliferative disease with strong oncogene, while there is a shift to more solid tumors with dampened signaling. Despite quantitative differences in signaling, RAS tropism in some tumors tracked with one mutation, indicating qualitative differences. Using RNASeq, we demonstrated unique transcriptional signatures appeared with lowest oncogenic signaling in comparison to the highest. Lower Kras activity induces a plastic state while high Kras signaling induces potent oncogenic activity and oncogenic stress. Our findings suggest potential unique vulnerabilities during tumor initiation driven by different RAS mutants.

MEK inhibitor resistance in lung adenocarcinoma is associated with addiction to sustained ERK suppression, representing a novel vulnerability

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MEK inhibitors (MEKi) have yielded limited efficacy in KRAS mutant lung adenocarcinoma (LUAD) patients, and this is attributed to both intrinsic and adaptive mechanisms of drug resistance. While many studies have focused on the former, there remains a dearth of data regarding acquired resistance to MEKi in LUAD. To address this, we established trametinib resistant KRAS mutant LUAD cells from initially sensitive cultures through dose escalation studies. Comparing the resistant cells to their sensitive counterparts though targeted next generation sequencing revealed alteration of both known and novel genes associated with trametinib response. Importantly, we described a state of “drug addiction” in resistant cases where cells are dependent on continuous culture in trametinib for survival. We show that dependence on ERK2 suppression underlies this phenomenon and that drug removal hyperactivates ERK resulting in ER stress and apoptosis. Amplification of mutant $KRAS^{G12C}$ occurs in drug addicted cells and blocking mutant specific activity with AMG-510 rescues the lethality associated with trametinib withdrawal. Lastly, we show that increased $KRAS^{G12C}$ expression is lethal to other KRAS mutant LUAD cells, which is also due to ERK hyperactivation. Overall, our data highlight mechanisms of adaptive resistance to MEKi in LUAD, revealing a potential drug addicted state in tumors that undergo KRAS amplification, presenting an avenue for new therapeutic strategies to improve patient response.

Deciphering IQGAP1 and IQGAP2 Scaffold Proteins Role on Melanoma Migratory and Invasive Capacities

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INTRODUCTION: Melanoma is a highly aggressive tumour that can metastasize very early in disease progression, being the skin cancer with the highest mortality rate.
Ras-ERK scaffold proteins can modulate MAPK signalling by assembling pathway components into multi-enzymatic complexes regulating signal intensity, amplitude and duration.
IQGAP1&2 are multi-domain scaffold proteins implicated in multiple biological processes including cell-cell adhesion, transcription and cytoskeletal architecture.

MATERIALS AND METHODS: Bioinformatic analyses of GEO and TCGA databases were performed to determine scaffold mRNA levels throughout melanoma progression. These results were supported *in vitro*, by immunoblot of paired melanoma cell lines with different metastatic potential. Moreover, proliferation, migration and invasion assays using 3D collagen matrices were performed to determine IQGAP1&2 relevance in melanoma spreading capacities.

RESULTS: Bioinformatic analyses revealed that mRNA scaffold levels were lower in melanocytes, in comparison with those in melanoma cells. Moreover, during melanomagenesis, a general enhance in scaffold mRNA levels was detected, while only some of them remained overexpressed in the metastatic stage. These results were confirmed *in vitro* by immunoblotting, being IQGAP2 levels higher in those cells derived from more advanced disease stages.
Besides, in melanoma cells, IQGAP1 and IQGAP2 were silenced by siRNAs being their proliferation, migration and invasive capacities decreased.

CONCLUSIONS: Ras-ERK scaffold proteins seem to play an important role in melanoma initiation, as their mRNA levels are increased in the previous stages of the disease. Moreover, IQGAP1 and IQGAP2 silencing in melanoma cells reduced their proliferation, migration and invasion capacities, making them a potential target for antitumoral drug development.

Decoding Mutant KRAS Signaling in Heterocellular Systems Using Interactomics

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Recent studies of the KRAS ^{G12Mut} interactome have highlighted multiple interactors that are critical to its oncogenic activity and may represent candidate therapeutic targets in PDAC. However, to date, these studies have focused on cells grown in monoculture, whereas cancer cells exist in a heterogeneous tumour microenvironment that is crucial to cancer progression and therapeutic resistance. This is exemplified in PDAC tumours, which possess a high degree of stromal content (80%).

In order to study KRAS^{G12Mut} specific interactors in PDAC, and determine if the KRAS^{G12Mut} interactome is dynamically modulated in the presence of other cell types, we are utilizing BioID and TurboID methodologies to identify KRAS^{G12D} interactors in heterocellular systems. Theses workflows allow for specific labelling of KRAS^{G12D} interactors in a cell-specific manner for subsequent identification via mass spectrometry.

Four KPC pancreatic cancer cell lines with distinct genetic, transcriptional and morphological characteristics were genetically engineered to express inducible KRAS^{G12D}-TurboID fusion proteins. In monoculture, these cell lines all displayed a core-set of KRAS^{G12D} interactors, as well as a cell line specific set of interactions. Further, upon co-culture with pancreatic associated fibroblasts (PaFs), a distinct interactome was uncovered indicating a significant input from PaFs on the KRAS interactome.

This data and methodology highlights the contribution of heterocellular signaling on the interactome of KRAS^{G12D} within tumour cells. Further development of this approach will allow for the characterization of the KRAS^{G12D} interactome and signaling space in native settings with the potential to highlight novel therapeutic targets in PDAC.

KRASG12D mutant cells are outcompeted by wild type neighbours in adult pancreas in an EPHA2-dependent manner.

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During ageing, tissues frequently accumulate genetic mutations in cancer-causing genes, yet cancer occurrence is a relatively rare event. We and others have previously shown that cells carrying genetic mutations in key oncogenes are outcompeted by normal neighbours, and are often eliminated from tissues, suggesting cell competition prevents tumourigenesis. However, the molecular mechanisms governing cell competition and the relevance of this process to cancer remain poorly understood. Using in vivo mouse models of pancreatic cancer and quantitative imaging approaches, we investigate how the adult pancreas maintains tissue health following sporadic expression of oncogenic KrasG12D, a key driver mutation in pancreatic cancer. We show that when present in tissues in low numbers, KrasG12D mutant cells are outcompeted by normal neighbours and eliminated from exocrine acinar, ductal epithelial and endocrine tissue compartments. We show that elimination of mutant cells requires a remodelling of exocrine and endocrine epithelia at the level of cell volume and cell-cell adhesions. We identify EphA2 receptor tyrosine kinase as an essential signal in the clearance of KrasG12D cells from all tissue compartments. In the absence of functional EphA2, KrasG12D cells are retained in tissues and this leads to the early appearance of premalignant intraepithelial neoplasia (PanINs) in tissues. Thus, EphA2-dependent cell competition drives elimination of KrasG12D mutant cells from adult pancreas tissues and this is tumour preventative. We propose that EphA2 signalling is an evolutionary conserved mediator of RAS-driven cell competition in epithelial tissues and that EphA2 is a novel tumour suppressor in pancreatic cancer.

PIK3C3 is an Obligate Haplo-insufficient Tumor Suppressor Gene in *KRAS*-driven Lung Adenocarcinoma

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Sequencing of lung adenocarcinoma (LUAD) revealed frequent (~47%) heterozygous loss or rare mutations of *PIK3C3*, which encodes the Class III PI3K hVPS34. hVPS34 forms complexes with hVPS15, BECLIN1, and other proteins to regulate autophagy, endocytosis, and mTOR, but its role in LUAD has not been explored. *In vitro* studies showed that hVPS34 mutants preferentially impaired BECLIN1/UVRAG-containing complexes, diminished autophagy, and cooperated with mutant *RAS* in transformation assays. To assay effects on tumor formation, we delivered Ad5-SPC-Cre intratracheally to LSL-*KRas*$^{G12D/+}$;*Pik3c3*$^{f/+}$ and LSL-*KRas*$^{G12D/+}$;*Pik3c3*$^{f/f}$ mice. Compared with LSL-*KRas*$^{G12D/+}$ mice, LSL-*KRas*$^{G12D/+}$;*Pik3c3*$^{f/+}$ mice developed more and faster growing tumor nodules and had shorter survival. *KRas*-mutant LUAD cells with *Pik3c3* heterozygous deletion also proliferated faster in 3D cultures. Cells from *KRas*$^{G12D/+}$;*Pik3c3*$^{f/+}$ tumors also proliferated faster in 3D culture. Similar results were obtained with compound mutants of *Pik3c3*$^{f/+}$ and *KRAS*$^{G12C/+}$ but not *Braf*$^{V600E/+}$. Consistent with the *in vitro* assays, heterozygous *Atg5* deletion also accelerated tumorigenesis in *KRas*$^{G12C/+}$ mice. By contrast, homozygous *Pik3c3* deletion restored tumor incidence and rate to that on parental *KRas*-mutant mice. Tissue-Mass Spectrometry identified 194 upregulated and 262 downregulated proteins in *KRas*$^{G12D/+}$;*Pik3c3*$^{f/+}$ tumor nodules. Upregulated proteins were highly enriched for immune-related and Wnt signaling pathways. TCGA data showed that patients with *KRAS* mutant/*Pik3c3*$^{+/-}$ LUAD had higher *WNT5A* and *WNT5B* expression. Our results indicate that *PIK3C3* is an obligate haplo-insufficient tumor suppressor gene in *KRAS*-mutant LUAD and suggest that incomplete autophagy inhibition might have deleterious therapeutic effects. By contrast, *PIK3C3* heterozygosity might be a biomarker for tumors that would be sensitized to autophagy inhibition.

Development of third generation Deltaflexins – K-Ras selective PDE6D inhibitors with potencies comparable to Deltazinone

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Oncogenic RAS mutations are found in ~19% of cancer patients with K-Ras4B (hereafter K-Ras) being the most frequently mutated isoform and a driver of cancer cell stemness. Since Ras proteins are difficult to target directly, alternative approaches are investigated. PDE6D is a trafficking chaperone for prenylated proteins and has been nominated as a surrogate target for K-Ras. By binding the farnesyl-moiety at the C-terminus of K-Ras, PDE6D promotes K-Ras diffusion in the cytoplasm and helps maintain K-Ras plasma membrane localization and activity.

Several high-affinity PDE6D inhibitors were developed. However, their cellular efficacy remained below expectation, mostly because the inhibitors were ejected from the PDE6D binding pocket by a GTP-Arl2/3-dependent mechanism. Our group has previously developed novel PDE6D inhibitors, called Deltaflexins, which harbor a ‘chemical spring’ to resist this ejection mechanism.

Our best third generation Deltaflexin shows the same in vitro activity against PDE6D as the previously developed Deltazinone. On-target activity in cells was demonstrated using Bioluminescence resonance energy transfer (BRET) biosensors to assess K-Ras vs. H-Ras selectivity and disruption of the PDE6D/ K-Ras interaction. In addition, we developed BRET-biosensors to determine off-target activity against UNC119a, a trafficking chaperone for myristoylated proteins that is highly related to PDE6D. While neither Deltaflexins nor Deltazinone inhibited the UNC119a/Src-interaction, Deltarasin displayed significant off-target activity, which may in part explain its broader toxicity. Furthermore, the anti-proliferative activity of Deltaflexins on a panel of cancer cell lines reflects the KRAS vs. HRAS mutant selectivity.

This study illustrates a significant potential for the improvement of PDE6D inhibitors.

Kinome dependencies in Ras/p53 mutant squamous cell carcinoma

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The oncogene RAS and the tumor suppressor gene TP53 are both frequently mutated in human cancer. Both *in vitro* and *in vivo* model systems have shown that mutations in Ras and p53 cooperate to generate a more malignant cell state. However, targeted therapies specific to tumors with mutations in RAS and/or TP53 are lacking. To identify novel druggable targets to cancers with mutations in Ras and p53, we performed arrayed, kinome focused siRNA and oncology drug phenotypic screening utilizing a set of syngeneic Ras mutant squamous cell carcinoma (SCC) cell lines that also carried co-mutations in selected p53 pathway genes. These cell lines were derived from SCCs from carcinogen treated inbred mice which carried germline deletions or mutations in *Trp53, Arf* ^{p19}*, Atm*, or *Prkdc*. Parallel siRNA screens were performed in the presence of doxorubicin to identify sensitizers and suppressor to this chemotherapeutic drug. Differences in functional kinome profiles between cell lines and after doxorubicin exposure indicate that both p53 pathway co-mutations and DNA damage rewire survival pathways in Ras mutant tumors. Both siRNA and drug profiling converge to implicate signaling through receptor tyrosine kinases, phosphoinositides, MEK, and mTOR, cell cycle regulation, DNA damage responses, and orphan kinases as targetable dependencies in SCC. This study describes the functional kinomic landscape of Ras/p53 mutant SCC in both the baseline unperturbed state and following DNA damage and nominates candidate therapeutic targets for further development.

Defining the KRAS and ERK-regulated transcriptome in *KRAS*-mutant pancreatic cancer

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Previous studies defined KRAS gene signatures with gene expression data of *KRAS*-mutant versus wildtype cancer cell lines or tumors and KRAS-transformed cell models. These signatures are used successfully to detect KRAS activity but are complicated by heterogeneity between samples or lack relevant mutation spectrums within cell models. To overcome these limitations, we used gene knockdown (KD) in a panel of *KRAS*-mutant pancreatic ductal adenocarcinoma (PDAC) cell lines. Cells were treated with *KRAS* siRNA for 24h to minimize compensatory signaling upon KRAS suppression. We identified 1008 and 699 genes that increased or decreased in expression, respectively, upon *KRAS* KD. We then examined independent RNA-seq data from pancreas cancer cell lines and micro-dissected pancreatic tumor epithelial cells. *KRAS* KD signature genes were twice as likely to be detected in these PDAC data than were genes of previously defined KRAS signatures. They also showed more evidence of epigenetic activation marks, increased DNase hypersensitivity, and increased Pol II occupancy in promoters (PANC-1 and normal pancreas), implicating epigenetic state in KRAS dependent gene expression. To determine RAF-MEK-ERK effector signaling network contributions to KRAS-dependent gene expression, we examined RNA-seq of PDAC cells treated with ERK-selective inhibitor SCH772984. *KRAS* KD and SCH772984 gene expression patterns were highly similar (PPV: 0.8). In summary, our findings demonstrate (1) significant tissue-type dependence of the KRAS transcriptome and (2) KRAS-regulated gene expression in PDAC is mediated largely through ERK. Ongoing studies will determine the contribution of upregulated genes in KRAS-dependent growth to identify novel therapeutic strategies for targeting KRAS in PDAC.

CHK1 protects oncogenic KRAS-expressing cells from DNA damage and is a target for pancreatic cancer treatment

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KRAS is a well-validated driver of the growth of pancreatic ductal adenocarcinoma (PDAC), largely through the ERK MAPK cascade. To identify genes essential for this growth, we applied several genetic screens to identify modulators of KRAS-mutant PDAC sensitivity to ERK-selective inhibitor SCH772984 (ERKi) treatment. Multiple components of the ATR-CHK1 DNA damage repair (DDR) pathway were identified. Pharmacological inhibition of CHK1 with prexasertib (CHK1i) alone potently blocked the growth of KRAS-mutant PDAC cell lines and patient-derived organoids (PDOs), and increased apoptosis at doses correlated with CHK1i induced S-phase arrest. Additionally, CHK1i decreased MYC expression but paradoxically activated ERK, providing a mechanistic basis for increased efficacy of CHK1i combined with ERKi. Interestingly, CHK1i also activated AMPK and increased autophagic flux. Combining CHK1i with the autophagy inhibitor chloroquine (CQ) caused increased growth suppression and apoptosis. Furthermore, triple combinations (CHK1i+ERKi+CQ) increased growth suppression and apoptosis even further in both cell lines and PDOs. To understand how activation of ERK promotes PDAC survival in the presence of CHK1 inhibition, we performed a CRISPR-Cas9 loss-of-function screen using a library targeting 1,223 direct/indirect ERK substrates. Loss of RIF1, a key component of non-homologous end-joining repair (NHEJ), sensitized PDAC cells to CHK1i mediated growth suppression and apoptosis. Furthermore, ERKi decreased RIF1 expression and either ERKi or RIF1 depletion reduced 53BP1 foci, which are essential for NHEJ repair, in combination with CHK1i. Overall, we demonstrate that targeting the DDR pathway enhances the efficacy of ERK and/or autophagy inhibitors in KRAS-mutant PDAC.

A pathogenic bacterium targets host NRas during infection

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Legionella pneumophila (Lp), an intracellular bacterial pathogen and causative agent of Legionnaire’s pneumonia, uniquely accumulates the human GTPase NRas, but not its homologs HRas or KRas, on the Lp containing vacuole (LCV). Mutagenesis indicates the NRas sites for farnesylation and palmitoylation are both required for NRas recruitment to the LCV. Interestingly, while HRas is not recruited to the LCV, a mono-palmitoylated HRas-C181S mutant (Golgi localized) was recruited, while mono-palmitoylated HRas-C184S (plasma membrane localized) was poorly recruited, suggesting that the selectivity for NRas recruitment depends on its unique Golgi enrichment. Active signaling was detected from LCV localized NRas using a fluorescently tagged Raf-1 Ras-binding-domain, suggesting a novel role for this GTPase in microbial pathogenesis and manipulation of host signaling pathways. The Lp effector responsible for NRas recruitment to the LCV, designated DenR, colocalizes with NRas during ectopic production in cells and causes a significant increase in NRas localization on internal membranes. By adding a Tom20-targeting sequence to DenR, NRas can be redirected to the mitochondria. These data corroborate pull-down experiments, suggesting DenR interacts with NRas to alter its subcellular localization. Together, these results provide the first evidence for an intracellular pathogen exploiting NRas GTPase function and reveal an unexpected link between NRas signaling and microbial infection. This work will shed light on the isoform-specific regulation of Ras proto-oncogenes, and ultimately enable rational drug design to target NRas-driven malignancies and pathogens that manipulate Ras GTPase function during infection.

The long noncoding RNA lnc-FANCI-2 in HPV-infected cervical cancer cells plays an important role in RAS signaling through MCAM

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We recently discovered the increased expression of cytoplasmic lnc-FANCI-2 along with cervical lesion progression from CIN1, CIN2-3 to cervical cancer. Viral E7 of high-risk HPVs and host transcription factor YY1 are two major factors promoting lnc-FANCI-2 expression (Liu H, et al. PNAS 118(3): e2014195118, 2021). To explore possible roles of lnc-FANCI-2 in HPV-induced cervical carcinogenesis, we knocked out lnc-FANCI-2 in an HPV16-positive cervical cancer cell line, CaSki cells, by using CRISPR-Cas9 technology. All lnc-FANCI-2 knock-out (KO) single cell clones expressed HPV16 oncogenes normally but displayed altered cell morphology when compared with wild-type (WT) parental cells. Proteome profiling of cytosolic and secreted proteins from WT cells and KO cells identified that lnc-FANCI-2 regulates expression of a subset of cell surface or adhesion-related proteins, including decreased MCAM, PODXL2 and ECM1 and increased ADAM8. RNA-seq analyses found significant increase of expression of the genes involved in RAS signaling pathways in the KO cells. Phosphorylated Akt and Erk, two important effectors of RAS pathway, were increased more than 3-fold, accompanied by ~2.9-fold increase of MCAM, a possible PI3K/AKT activator, in KO cells over WT cells. In parallel, we showed that siRNA-knockdown of lnc-FANCI-2 expression in WT CaSki cells also increased MCAM by 87%, phosphorylated Akt by 54%, and phosphorylated Erk2 by 43%, over the non-specific siRNA control cells. Simultaneous siRNA knockdown of the increased MCAM expression in the lnc-FANCI-2 silenced cells blocked the increased phosphorylation of Akt and slightly reduced p-Erk2. Interestingly, the high-level of lnc-FANCI-2 expression and the lower level of MCAM expression in the patients with cervical cancer were found being associated with a much better survival rate ($P<0.001$). Altogether, we have discovered that lnc-FANCI-2, a host long noncoding RNA, restricts RAS signaling by regulation of MCAM expression.

Exon14-skipping mutations in the Hepatocyte Growth Factor Receptor confer oncogenicity via preferential activation of the Ras/MAPK pathway

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Splice site mutations in the hepatocyte growth factor receptor (MET) resulting in loss of exon14 (METΔex14) constitute of group of known driver mutations in lung adenocarcinomas. Though MET is frequently overexpressed in various cancers, its increased expression is rarely sufficient to drive tumourigenesis alone, suggesting METΔex14 confers additional transformative properties. By comparing the transcriptional profiles of MET-addicted LUAD tumours and cell lines with METΔex14 mutations vs. those with MET overexpression only, we found RAS/MAPK pathway target genes to be upregulated in METΔex14 samples. Furthermore, in METΔex14 cell lines, expression levels of these genes were found to correlate with MET receptor activity. By contrast, we failed to observe a similar pattern in the MET WT amplified line. In an isogenic system comparing signalling between WT and dEx14 mutants *in vitro*, overexpressing METΔex14 in lung epithelial cells resulted in a higher fraction of active RAS compared to overexpressing MET WT. Furthermore, similar analysis in HEK293 cells show an ability for the METΔex14 receptor to drive differentially higher levels of MAPK phosphorylation compared to its MET WT overexpressing counterpart. We did not observe similar differential activity in parallel PI3K/Akt or Stat3 pathways, ruling out general hyperactivity of the METΔex14 receptor as a culprit. In agreement, siRNA knockdown of RAS in these cells had a greater impact on viability in METΔex14 cells compared to MET WT-expressing cells. Our results indicate that METΔex14 mutations confer on cells a greater ability to activate the MAPK pathway via RAS, an important pathway for cell survival and proliferation.

A Proteomic Approach Reveals RAS Heterodimerization.

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Active mutations in the *RAS* genes are found in ~20-30% of human cancers. Although thought to have overlapping functions, Ras isoforms show preferential activation in different types of human tumors. This prompted us to employ a comparative and quantitative proteomics approach to generate isoform-specific and nucleotide-dependent interactomes of the four Ras isoforms, KRAS4a, KRAS4b, HRAS, and NRAS. We originally thought that targeting these isoform-specific interacting proteins could selectively target cells transformed with the corresponding Ras isoform. Interestingly, when performing a synthetic lethal screen using shRNA and small molecule inhibitors, such selectivity was not observed. In attempt to rationalize this, we noticed that our proteomic data indicated different isoforms of Ras could interact with each other. Using crosslinking and FRET, HRAS and KRAS were found to form heterodimers in cells. Using a reported homodimer impairing mutation, D154Q, reduction of the heterodimer formation was also observed. The heterodimer formation could explain the lack of Ras isoform specificity when targeting isoform-specific interactors. Although Ras homodimers have been reported, to our knowledge, the formation of heterodimer had not been previously demonstrated.

Identification of KRAS suppressor genes: An ORFeome library screening

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The RAS family genes encode small GTP-binding cytoplasmic proteins that are important signaling molecules. They regulate cell growth, survival and differentiation by coupling receptor activation to downstream effector pathways. Activating mutations of oncogenic RAS pathway genes are frequently detected in human cancers. KRAS is the most frequently mutated oncogene in human. Most of these tumors are addicted to KRAS signaling. However, upon treatment with RAS signaling pathway inhibitors, KRAS signaling addiction is often lost. Tumors can become resistant through the activation of suppressor mechanisms. A possible explanation is that these tumors carry a second genomic perturbation that can compensate for the detrimental effects of the RAS signaling inhibitors, a phenomenon referred to as suppression. In model organisms, suppression interactions are generally divided into two classes: genomic suppressors which are secondary mutations in the genome that bypass a mutant phenotype, and dosage suppression interactions in which overexpression of a suppressor gene rescues a mutant phenotype. We decided to use the latter strategy in human cancer cells to identify KRAS suppressor genes. KRAS suppressor genes can be involved in resistance mechanisms of RAS pathway inhibitors and this approach could identify new targets for drug research programs and/or novel drug combinations.

In this study we performed a screen on a human Open Reading Frame library (hORFeome collection) in order to identify potential KRAS suppressor genes. Our screening strategy, our first results as well as our first identified suppressors will be presented.

ERK2 drives MYC expression acting as a kinase-independent anchor for CDK9 at the MYC promoter

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Over the last years, it has been widely demonstrated that ERK is able to bind to DNA and modify gene expression. Here, we present evidence that ERK directly regulates *MYC* transcription, a process that, until now, was largely unknown. Bioinformatic analyses showed that ERK binds to the *MYC* promoter at a defined motif: C/GAAAG/C, and we confirmed this by ChIP in human 293T cells and mouse embryo fibroblasts (MEFs), where we detected the presence of ERK in the promoter regions with ERK conserved boxes. Moreover, we demonstrated that this recruitment is sufficient to induce synthesis of *MYC* at mRNA and protein levels. Another partner in this process is CDK9. We have confirmed that CDK9 and ERK are present in the same region of the *MYC* promoter and that both proteins directly interact thought the CD domain of CDK9. We have established that this interaction allows ERK to induce *MYC* expression via CDK9 and that in all of these processes ERK is acting in a kinase-independent manner. Overall, our results uncover an unprecedented role for ERK, acting as a kinase-independent anchor for the recruitment of CDK9 to the *MYC* promoter to regulate its expression.

Non Cell-Autonomous Cooperation of Activated RAS and MYC Drives Aggressive Tumors

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Intratumor genetic heterogeneity presents a major clinical challenge for successfully targeting therapeutic vulnerabilities in solid cancers. Although tumor heterogeneity has traditionally been thought to arise as an evolutionary mechanism of subclonal competition, our data supports an alternative model, in which subclonal cooperation facilitates productive interactions between distinct subclones to promote more aggressive tumor growth. Activated RAS is a potent oncogene which can drive tumorigenesis across multiple tumor types, yet whether other oncogenic drivers in adjacent cells can cooperate with RAS driven subclones remains poorly understood.

We developed an isogenic system to study how distinct oncogenic drivers can interact within the context of a complex tumor. We used HRAS ($HRAS^{G12V}$) transformed MCF10A cells and those overexpressing MYC as exemplars to study tumor heterogeneity. Using 3-dimensional spheroid culture, we modeled the multicellular structure of a biclonal tumor in cell culture, demonstrating that MYC increases the proliferative capacity of HRAS when grown in admixed spheres compared to HRAS monoclonal cultures. Most importantly, we observed a dramatic increase in the tumorigenic potential of HRAS cells *in vivo* to induce tumor formation when co-injected with MYC cells, resulting in more rapid and aggressive tumor formation compared to HRAS-only controls. Finally, we performed RNA-seq on MYC and HRAS populations, grown respectively in admixed or monoclonal spheroid culture, to gain mechanistic insight into the increased tumorigenicity observed. This work will provide valuable insights into the functional significance of tumor heterogeneity and explore changes in the tumorigenic phenotypes of HRAS driven subclones of complex tumors.

The Dynamics of Kras Dimerization Unravels Potential Mechanism for Suppression of Hyperactive KRas4b

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Ras dimers have been proposed to be building blocks for initiating the ERK/MAPK cellular signaling pathway. However, little is known about whether and how Ras dimerizes. To examine the dynamics of Ras dimerization and its potential signaling consequences, we performed molecular dynamics simulations totaling 1 millisecond of sampling, using an all-atom model of two full-length farnesylated GTP-bound KRas4b proteins diffusing on 29%POPS-mixed POPC membranes. Our simulations unveil the formation of thermodynamically weak KRas dimers. We found that the HVR of each KRas have frequent interactions with various parts of the KRas dimer, thus potentially mediating Ras dimerization. Some rare KRas-dimer configurations have one KRas G-domain elevated above the lipid bilayer surface by residing on top of the other KRas, thus likely exposing more to cytosolic Raf kinases. Interestingly, we identified nine cationic residues that intermittently interact with the beta-phosphate of the GTP bound in KRas4b, in a manner similar to what is observed in a crystal structure of HRas, which have the Arginine finger of GTPase-activating protein (GAP) pointed toward the beta-phosphate. Specifically, KRas dimer conformations can result into GTP hydrolysis with R68 and R149 fingers, thus potentially inactivating itself. This KRas-dimer self-inactivating mechanism can be used to explain experimental observations that overexpressed KRas can inhibit the ERK activity and suppress tumor growth. The hydrolysis mechanism observed for the simulated KRas dimer suggests a strategy to control hyperactive KRas in tumor cells.

Comparing the motions of the KRAS4b:RAF1 complex in solution and at the membrane

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In the MAPK signaling pathway, the RAS-binding domain (RBD) and cysteine-rich domain (CRD) of RAF kinases bind to activated KRAS4b at the cell membrane, which initiates the RAS-driven cell proliferation. To elucidate the molecular nature of KRAS4b-RAF1 engagement and the role of the membrane, we perform extensive molecular dynamics (MD) simulations of the KRAS4b:RBD-CRD complex in solution, as well as in the context of plasma membrane mimetic. These calculations are carried out using all-atom (AA) explicit-solvent and coarse-grained (CG) forcefields. Specifically, our AA simulations describe the detailed interactions of the proteins and membrane, providing a foundation for us to build an accurate CG model that captures RAS-RAF dynamics at longer time scales. Through combined use of AA and CG simulations, we uncover the motions of CRD within the KRAS4b:RBD-CRD complex in solution, and demonstrate that CRD movement is significantly restricted when KRAS4b is anchored to the membrane. This comparison dissects the role of the membrane during this process. We show that our simulated ensembles are consistent with previous experimental and computational studies, and discuss how the new findings fill the gaps in our current understanding of RAS-RAF biology.

GAP Positions Catalytic H-Ras Residue Q61 for GTP Hydrolysis in MD Simulations

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Ras binds to a GTPase activating protein (GAP) to turn off the cell propagation signal. Together, they form a transition state that has a very precise configuration that catalyzes GTP hydrolysis. GAP provides an essential arginine residue to the transition state. Fluorescence experiments show that an arginine analogue alone cannot restore Ras function. We hypothesize that the shared interface between the proteins plays a critical role in the Ras-GAP transition state. We perform molecular dynamics simulations of the Ras-GAP complex and of Ras to do a comparison of Ras structural features and conformational freedom. We will show that GAP significantly restrains the switch I and II regions of Ras. Furthermore, the Ras Q61 residue that is mechanistically essential for GTP hydrolysis, is held closer to the GTP gamma phosphate in the Ras-GAP complex. This proximity may promote more frequent fluctuations into configurations that enable GTP hydrolysis.

Scaffold association factor B (SAFB) is required for expression of prenyltransferases and RAS membrane association

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Inhibiting membrane association of RAS has long been considered a rational approach to anti-cancer therapy which led to the development of farnesyltransferase inhibitors (FTIs). However, FTIs proved ineffective against *KRAS*-driven tumors. To reveal alternative therapeutic strategies we carried out a genome-wide CRISPR-Cas9 screen designed to identify genes required for KRAS4B membrane association. We identified five enzymes in the prenylation pathway and SAFB, a nuclear protein with both DNA and RNA binding domains. Silencing *SAFB* led to marked mislocalization of all RAS isoforms as well as RAP1A, but not RAB7A, a pattern that phenocopied silencing *FNTA*, the prenyltransferase α subunit shared by farnesyltransferase and geranyl-geranytransferase type I. We found that SAFB promoted RAS membrane association by controlling FNTA expression. *SAFB* knockdown decreased GTP-loading of RAS, abrogated alternative prenylation and sensitized *RAS*-mutant cells to growth inhibition by FTI. Our work establishes the prenylation pathway as paramount in KRAS membrane association, reveals the first regulator of prenyltransferase expression and suggests that reduction in FNTA expression may enhance the efficacy of FTIs.

A data-driven approach to predicting Ras/MAPK pathway activation with downstream target genes.

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Ras activation stimulates Mitogen Activated Protein Kinases (i.e. ERK) and leads to phosphorylation and transcription of downstream target genes. In many studies, activation of the Ras/MAPK pathway is often assayed by immunostaining for phosphorylated ERK or its elevated expression of downstream target genes. However, recent studies take advantage of live cell reporter technology to assay real-time pathway activity *in vitro* and *in vivo*. While these reporters reveal detailed spatiotemporal signaling information, immunostaining is much simpler to implement when a quick inference about recent Ras pathway activity is needed. In this study, we combine a live-cell reporter approach with multiplexed immuno-fluorescence staining of downstream target proteins to ask how well immunostaining captures recent history of activity in mammary epithelial cells. We fit regression models to reveal which target proteins most correlate to activated Ras signaling, and identify the staining patterns that best predict high, low, or variable pathway activation.

Translational control of the Epithelial-to-Mesenchymal Transition (EMT) in Ras-driven colorectal cancer

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Our data reveal a novel role of a Ras-mediated molecular scaffold, Kinase Suppressor of Ras 1 (KSR1)-dependent ERK signaling to promote the preferential translation of Epithelial-Stromal Interaction 1 (EPSTI1), which is required to induce a switch from E- to N-cadherin and coordinate migratory and invasive behavior in colon cancer cell lines and colon tumor organoids. Activating Ras mutations, present in over 50% of colorectal cancer (CRC) cases, are critical to tumor growth and survival. KSR1 is a molecular scaffold that coordinates signaling through the Ras/Raf/MEK/ERK kinase cascade to facilitate maximum signal transmission. EMT, traditionally considered a transcriptional process, induces cells to switch from a polarized state to a migratory phenotype. When aberrantly activated, EMT has pathogenic effects contributing to malignant tumor progression and metastasis. By combining gene targeting of key effectors of Ras signaling with genome-wide polysome profiling, we discovered that KSR1 promotes the translation of proteins essential for EMT in Ras-mutant CRC cell lines. One preferentially translated protein, EPSTI1, induces a cadherin switch by coordinating the E-cadherin's downregulation and N-cadherin's upregulation. EPSTI1 is overexpressed in human CRC cell lines and colon tumor organoids compared to normal cells and is required for cancer cell survival. Disruption of KSR1 or EPSTI1 significantly impairs cell migration and invasion *in* vitro and reverses EMT, in part, by decreasing the expression of N-cadherin and the transcriptional repressor of E-cadherin expression, Slug. In CRC cells lacking KSR1, ectopic EPSTI1 expression restored the E- to N-cadherin switch, migration, invasion, and anchorage-independent growth. KSR1-dependent induction of EMT via selective translation of mRNAs reveals its underappreciated role in remodeling the CRC cells' translational landscape to promote their migratory and invasive behavior. Characterization of EPSTI1 repurposed by dysregulated translation in CRC should reveal novel mechanisms critical to CRC tumor survival and progression.

Functional analysis of the role of RAP1GDS1 and RhoA in KRAS-driven lung adenocarcinoma (LUAD)

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A combination of proteomics and CRISPR/Cas9 screens in human LUAD cells allowed to identify a KRAS-specific vulnerability induced by loss of the long isoform of RAP1GDS1 and RhoA, suggesting a potentially novel approach for targeting oncogenic KRAS. Here we use biochemical, proteomic and genetic approaches to dissect the isoform-specific roles of RAP1GDS1 to elucidate its synthetic lethal interaction with RhoA in KRAS-driven LUAD.

We performed AP/MS in A549 KRAS-mutant cells using both the long (RAP1GDS1-607) and the short (RAP1GDS1-558) isoforms of RAP1GDS1 to identify overlapping and isoform-specific RAP1GDS1 interactors. The analysis was enriched by overlapping our results with orthologous datasets: DepMap, published PPIs (Huttlin *et al*. and Nissim *et al*.) and an analysis of RAP1GDS1-607 loss on prenylation (Brandt *et al*). This analysis identified a cluster of RAB-GTPases that bind specifically to RAP1GDS1-607, suggesting endosomal RABs could mediate its oncogenic effect. We are currently characterizing the functional importance of these interactors using genetics and cell biology. We also used live cell imaging in H23 KRAS-mutant cells transfected with siRNAs against the different isoforms of RAP1GDS1 to determine how their loss change the subcellular localization of GFP-tagged KRAS and other RAP1GDS1 interactors. Lack of both RAP1GDS1-isoforms and, to a lesser extent, single loss of RAP1GDS1-607, decreased KRAS localization at the cell membrane. Finally, to identify the molecular mechanism of RAP1GDS1/RhoA synthetic lethality with KRAS, we performed a Genome Wide-CRISPR screen in RAP1GDS1-607 and RhoA knock-out cells. It allowed to find genes that are synthetic lethal together with RhoA or RAP1GDS1-607 and could mediate the lethal effect of RAP1GDS1-607/RhoA loss. We are using functional/biochemical assays to validate the most promising interactions of RAP1GDS1-607 or RhoA with key proteins likely to be important for the synthetic vulnerability we have observed.

In conclusion, this study will dissect the interplay between RhoA and RAP1GDS1-607 and identify new combinatorial dependencies.

The L57V mutation in the RAS-homologous GTPase RHOA is a distinct oncogenic alteration in diffuse gastric cancer

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Missense mutations of the RAS homologous RHOA gene were identified in 15-26% of diffuse gastric cancer (DGC). Surprisingly, these mutations are found at amino acid positions that are distinct from the mutational hotspots found in RAS. Consequently, whether RHOA mutations in cancer act as gain- or loss-of-function mutations remains unresolved. Here, we characterized the L57V mutation, the second most common RHOA alteration in DGC, after RHOA^{Y42C}. We found that RHOA^{L57V}, similar to RHOA^{Y42C} and the lab-generated mutant RHOA^{Q63L}, induced actin stress fibers, supporting a gain-of-function phenotype. Like RHOA^{Y42C}, RHOA^{L57V} disrupted gastric organoid architecture and induced a DGC-like phenotype. To determine the biochemical consequences of the L57V mutation, we utilized recombinant proteins and found that intrinsic and p190RhoGAP-stimulated GTP-hydrolysis were reduced in RHOA^{L57V} compared to WT. Therefore, like RHOA^{Y42C}, RHOA^{L57V} is activated partly through impaired GTPase activity. Also like RHOA^{Y42C}, RHOA^{L57V} retained WT intrinsic and RhoGEF (ECT2)-catalyzed nucleotide exchange activity. However, in contrast to RHOA^{Y42C}, RHOA^{L57V} did not exhibit impaired effector binding. Further, reverse phase protein array (RPPA) signaling analyses identified IGF1R and PAK1 activation in organoids expressing RHOA^{L57V} but not RHOA^{Y42C}. Finally, we evaluated the oncogenic potential of mutations in RAS that are analogous to RHOA cancer-associated alterations. Consistent with their absence in cancers, mutations at these positions did not exhibit significant gain-of-function activities, further distinguishing how RHOA and RAS oncogenic potential is unmasked. In summary, both RHOA^{L57V} and RHOA^{Y42C} induced an oncogenic phenotype leading to DGC, but through distinct biochemical and signaling mechanisms.

Defining the role of MYC in KRAS-driven pancreatic cancer

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Despite a well-established genetic landscape, current standards of care in pancreatic ductal adenocarcinoma (PDAC) treatment are limited to cytotoxic chemotherapy as clinically effective anti-KRAS therapies remain elusive. One promising approach involves inhibition of a key KRAS effector pathway, the RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cascade. However, ERK can phosphorylate over 1300 substrates, and the crucial gene targets that mediate ERK-driven PDAC growth remain largely undefined. Substantial experimental evidence supports the MYC transcription factor and oncoprotein as a key ERK substrate mediating KRAS-driven PDAC. However, the mechanistic contribution of MYC remains an open question. To address this, I have initiated a comprehensive evaluation of the specific contributions of MYC in supporting KRAS-driven PDAC. First, RNA-Seq analyses demonstrated that KRAS depletion or ERK inhibition globally suppressed the MYC transcriptome, supporting a significant block in MYC function upon loss of KRAS-ERK signaling. Second, acute *KRAS* suppression in PDAC caused significant cell enlargement and flattening, enhanced actin stress fiber organization, and expression of proteins associated with a mesenchymal to epithelial transition. These changes were largely phenocopied upon MYC suppression. Third, applying reverse phase protein array pathway activation mapping to *KRAS* or *MYC* siRNA-treated PDAC cell lines, we observed overlapping alterations in signaling networks. However, several differences between KRAS and MYC loss were also observed. Finally, RNA-Seq analysis of the KRAS- versus MYC-dependent transcriptome demonstrated both significant overlap and key differences. In summary, my studies support a key role for MYC in facilitating diverse KRAS-driven cellular activities, and additionally, a role for MYC-independent processes.

The ETS Transcription Factor ERF controls the exit from the naïve pluripotent state

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The naïve epiblast undergoes a transition to a pluripotent primed state during embryo implantation. Despite the relevance of the FGF pathway during this period, little is known about the downstream effectors regulating this signaling. Here, we examined the molecular mechanisms coordinating the naïve to primed transition by using inducible ESC to genetically eliminate all RAS proteins. We show that differentiated RAS^{KO} ESC remain trapped in an intermediate state of pluripotency with naïve-associated features. Elimination of the transcription factor ERF overcomes the developmental blockage of RAS-deficient cells by naïve enhancer decommissioning. Mechanistically, ERF regulates NANOG expression and ensures naïve pluripotency by strengthening naïve transcription factor binding at ESC enhancers. Moreover, ERF negatively regulates the expression of the *de novo* methyltransferase DNMT3B, which participates in the extinction of the naïve transcriptional program. Collectively, we demonstrated an essential role for ERF controlling the exit from naïve pluripotency during the progression to primed pluripotency.

RAS specific protease Induces Irreversible Growth Arrest via p27 in several KRAS Mutant Colorectal Cancer cell lines

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Ras-specific proteases to degrade RAS within cancer cells are under active development as an innovative strategy to treat tumorigenesis. The naturally occurring biological toxin effector called RAS/RAP1-specific endopeptidase (RRSP) is known to cleave all RAS within a cell, including HRAS, KRAS, NRAS and mutant KRAS G13D. In the course of studies developing RRSP as an anti-cancer therapeutic, it was shown that cleavage of RAS by RRSP results in inhibition of tumor growth in breast and colon xenograft mouse models. Despite the considerable amount of evidence demonstrating RRSP anti-tumor effects in vivo, our understanding of the mechanisms involved are unknown. Here, we first demonstrate, using isogenic mouse fibroblasts expressing a single isoform of RAS or mutant KRAS, that RRSP equally inactivates all isoforms of RAS as well as the major oncogenic KRAS mutants. To investigate how RAS processing might lead to varying outcomes in cell fate within cancer cells, we tested RRSP against four colorectal cancer cell lines with a range of cell fates. While cell lines highly susceptible to RRSP (HCT116 and SW1463) undergo cytotoxic death, RRSP treatment of GP5d cells induces G1 cell cycle arrest, and SW620 cells instead induces growth inhibition through cell senescence. In three of four cell lines tested, growth effects were dictated by rescued expression of the tumor suppressor protein p27 (Kip1). The ability of RRSP to inactivate all RAS and inhibit cancer cell growth through a variety of mechanisms highlights the antitumor potential of RRSP, and further warrants investigation as a potential anti-tumor therapeutic.

Multiplexed identification of RAS paralog imbalance as a driver of lung cancer growth

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Oncogenic KRAS mutations occur in approximately 30% of human lung adenocarcinoma. Despite tremendous effort over the past several decades, KRAS-driven lung cancer remains difficult to treat and our understanding of positive and negative regulators of RAS signaling is incomplete. To uncover the functional impact of diverse KRAS-interacting proteins on lung cancer growth *in vivo*, we harnessed the power of affinity purification/mass spectrometry (AP/MS) and somatic CRISPR-based genome editing following by high-throughput barcode sequencing (Tuba-seq). We identified both HRAS and NRAS as suppressors of $KRAS^{G12D}$-driven lung cancer growth *in vivo*. Wild type HRAS and NRAS also suppressed growth of oncogenic KRAS-driven human lung cancer cell lines. Mechanistically, RAS paralogs interact with oncogenic KRAS, suppress KRAS-KRAS oligomerization, and reduce downstream MEK-ERK signaling. Rare $HRAS^{T50M}$ and $HRAS^{R123C}$ mutations from KRAS-mutant patients are dimerization deficient mutations which abolished this effect. At last, via paired screen in both $KRAS^{G12D}$-driven $BRAF^{V600E}$-driven lung cancer models, we confirmed that wild type HRAS and NRAS are specific suppressors of oncogenic KRAS-driven lung cancer *in vivo*. Our study outlines a technological avenue to positive and negative regulators that are specific to oncogenic KRAS-driven cancer in a multiplexed manner and highlights the role of RAS paralog imbalance in oncogenic KRAS-driven cancers.

Oncogenic KRAS signaling in luminal epithelial cells can give rise to basal-like and claudin-low mammary cancers

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Basal-like and claudin-low mammary tumors are the two most common molecular subtypes among triple-negative breast cancers. While it has been previously proposed that basal-like mammary cancers may arise from luminal progenitors in patients with BRCA1-associated breast tumors, it is thought that mammary stem cells are the cellular origin of claudin-low cancers that possess mesenchymal characteristics. Oncogenic drivers have yet to be defined, but recent work suggests that hyperactive RAS signaling may play a critical role in the genesis of claudin-low breast cancers. To experimentally address the biological significance of oncogenic RAS, we generated two new genetically engineered mouse models that express exogenous and endogenous oncogenic KRAS in the mammary gland epithelium in a constitutive and differentiation stage-independent manner. We demonstrate that preneoplastic luminal epithelial cells gain characteristics of basal epithelial cells that evolve into basal-like and claudin-low mammary cancers. Moreover, we show that the mesenchymal properties of claudin-low tumor cells are being continuously upheld by oncogenic KRAS and regulators of EMT that may function in a KRAS-independent manner.

RAS Senses Membrane Curvature in an Isoform-Specific Manner

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Tumors possesses distinct mechanical properties that contribute to the distinct morphologies of tumor cells and correlate with tumor growth. In mouse tumor allografts, melanoma cells expressing oncogenic mutant KRAS4B display an epithelial-like (flat and round) morphology, while inhibiting oncogenic KRAS function turns the cells to an elongated morphology. Prostate cells expressing active HRAS possess an elongated morphology and become rounded when HRAS signaling is perturbed. Thus, RAS may play isoform-specific roles in the plasma membrane (PM) curvature-sensing in cancer cell mechanotransduction. We integrated quantitative super-resolution imaging and biophysical assays, and directly compared the PM curvature sensing of RAS mutants, KRAS4B^{G12V} vs. HRAS^{G12V}. In synthetic vesicles, isolated PM blebs and intact/live cells, we observed consistent RAS isoform specificity: flatter membranes with less curvature enhanced the nanoclustering and signaling of KRAS4B^{G12V}, but disrupted those of HRAS^{G12V}. The nanoclustering of KRAS4B^{G12V} and HRAS^{G12V} also responds to curvature directions in distinct manners. Specifically, positive membrane curvature disrupted the nanoclustering of KRAS4B^{G12V}, but promoted the clustering of HRAS^{G12V}. On the other hand, negative membrane curvature had no effect on KRAS4B^{G12V}, but perturbed the nanoclustering of HRAS. Further, depleting an anionic phospholipid, phosphatidylserine (PS), abolished the membrane curvature sensing of KRAS4B^{G12V}, which was only restored by the acute addback of mixed-chain PS species, but not other PS species tested.

This novel membrane curvature-sensing capability potentially contributes to the correlation between RAS oncogenesis and tumor cell morphology and mechanics. Membrane curvature sensing may also contribute to the distinct intracellular trafficking patterns of different RAS isoforms.

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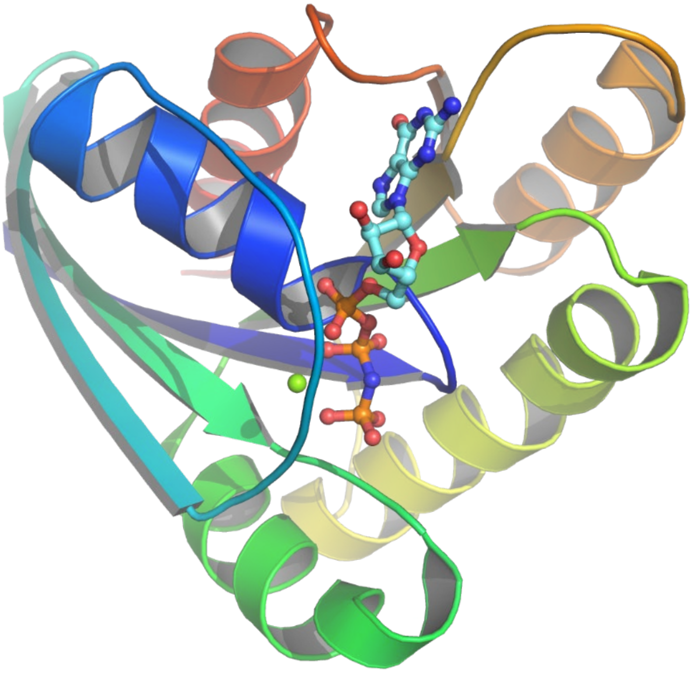

Structure

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Characterizing the effect of lipid composition on the RAS-RAF complex

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The human body is made of trillion of cells, each programmed to perform essential processes, which are carried out by signaling cascades passing through the cell membranes, that act as control barriers and signaling platforms. Cell membranes have an incredibly complex composition, making them extremely difficult to simulate using conventional molecular dynamics simulations. Multiscale molecular dynamics simulations is a novel technique that uses continuum models to mimic real time processes of complex systems, while avoiding compromises in the intricacy of its composition and fluidity. These continuum models can be broken down into patches and analyzed using coarse grained and all-atom approaches. RAS is a membrane protein that is part of the signaling cascade responsible for cell growth and localized mutations in RAS account for 20% of cancer cases. When RAS is mutated, it remains in its active state, propagating the growth signal to its effector protein, RAF, and causing uncontrollable cell growth. In our project, we make use of this novel technique to study the effect of lipid composition on the signaling cascade of the RASRAF complex. Our hypothesis is that lipid composition has a direct effect on the orientation of RAS and its capability to bind to RAF to propagate the signal. We were able to get insight into how lipid composition influences signaling cascades that propagate through the membrane. Our better understanding of this interaction would help us make informed developments towards new ways to switch off the signaling cascade and stop cell growth in cancer cells.

High-resolution cryo-EM structure of full-length NF1

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Neurofibromin (NF1) is a GTPase-activating protein (GAP) and regulates the GTPase Ras, thus acting as a tumor suppressor. As a result, NF1 mutation and dysregulation is central to many diseases including neurofibromatosis, melanoma, leukemia and lung cancer. Understanding the structure-activity relationship of NF1 is crucial to developing effective therapeutic strategies to combat these diseases. While NF1 has been previously identified as a high-affinity dimer, high-resolution structural information of NF1 is currently limited to only the GAP related domain (GRD) and phospholipid binding domain (Sec-PH), encompassing around 20% of the protein. Thus, a more detailed and complete structure of the full-length protein is needed, to elucidate the chemical interactions that contribute to NF1 structural integrity and function, as this would provide mechanistic insights and serve as a guide to interpret the effects of many disease-associated mutations. In this presentation, we describe the cryo-EM structure of full-length NF1 and highlight important structural details.

The structure of membrane-bound KRas from experiment and simulation.

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KRas4B is a membrane-anchored signaling protein and primary target in cancer research. Predictions from molecular dynamics simulations have shaped our mechanistic understanding of KRas signaling but disagree with recent experimental results from neutron reflectometry, nuclear magnetic resonance, and thermodynamic binding studies [1]. We compare this body of biophysical data to back-calculated experimental results from a series of molecular simulations that implement different subsets of molecular interactions. Our results show that KRas4B approximates an entropic ensemble of configurations at model membranes, which is not significantly affected by interactions between the globular G-domain of KRas4B and the lipid membrane. These findings promote a model of KRas, in which the G-domain explores the entire accessible conformational space while being available to bind to effector proteins.

[1] Van, Q. N. *et al.* Uncovering a membrane-distal conformation of KRAS available to recruit RAF to the plasma membrane. *Proc National Acad Sci* **117**, 24258–24268 (2020)

The Ras Dimer Structure

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The Ras dimer interface is a potential drug target. *In vivo* studies suggest that preventing dimerization of oncogenic mutant Ras inhibits uncontrolled cell growth. Conventional computational drug screening approaches require an accurate atomic dimer model as input to successfully access drug candidates. However, the proposed dimer structural models are controversial. We present a clear-cut experimentally validated N-Ras dimer structure model. We incorporated unnatural amino acids into Ras to enable the binding of labels at multiple positions via click chemistry. This labeling allowed determination of multiple distances within membrane-bound Ras dimers as measured by fluorescence and electron paramagnetic resonance spectroscopy. In combination with protein-protein docking and biomolecular simulations, we identified key residues for dimerization. Site-directed mutations of these residues prevent dimer formation in our experiments, proving the correctness of our dimer model. The presented dimer structure now enables computational drug screening studies using the Ras dimer interface as an alternative drug target.

P negatively regulates RAF kinase activity and 14-3-3 induced RAF dimerization overcomes this effect

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BRAF is a protein kinase that drives cell proliferation through a phosphorylation cascade of downstream kinases MEK and ERK. As one of the most mutated proteins in human cancers, BRAF continues to be an important target of research for cancer therapies.
Dimerization is known to be crucial for the upregulation of BRAF catalytic activity. We have solved an X-ray crystal structure of a BRAF kinase domain ($BRAF^{KD}$) in complex with MEK1 and the ATP analog AMP-PCP (ACP), which surprisingly reveals a monomeric form of $BRAF^{KD}$ in a catalytically inactive state. This novel conformation of BRAF is incompatible with dimerization due to a twisting of the N-lobe of the kinase domain with respect to its C-lobe. ACP is also able to break RAF^{KD} dimers in solution.
The 14-3-3 proteins are constitutive dimers that are also known to be necessary for activation through the interaction with a phosphorylated C-terminal motif on BRAF, though the molecular mechanism for such activation was unknown until recently. We have solved X-ray crystal structures of a $BRAF^{KD}$-$BRAF^{KD}$ homodimer in the active conformation, bound to a 14-3-3 dimer in both the presence and absence of ligands in the ATP binding site. Our biochemical experiments show that ligand binding to the ATP site in the BRAF dimer is symmetric and that cellular ATP concentrations lower BRAF dimer affinity and kinase activity. Overall, our results reveal how 14-3-3 binding is both necessary and sufficient to overcome the negative regulatory effect of ATP, without the need for the engagement of other regulatory elements of BRAF.

The Mechanism of Activation of Monomeric B-Raf V600E

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Oncogenic mutations in the serine/threonine kinase B-Raf, particularly the V600E mutation, are frequent in cancer, making it a major drug target. Although much is known about B-Raf's active and inactive states, questions remain about the mechanism by which the protein changes between these two states. Here, we utilize molecular dynamics to investigate both wild-type and V600E B-Raf to gain mechanistic insights into the impact of the Val to Glu mutation. We seek to understand why B-Raf V600E is constitutively active in its monomeric state while wild type B-Raf requires dimerization. The results show that the wild-type and mutant follow similar activation pathways involving an extension of the activation loop and an inward motion of the αC-helix. We see that when the activation loop is extended, the αC-helix can move between an inward and outward orientation with a corresponding formation and breaking of a salt-bridge between Lys483 and Glu501. This motion is possible only when the DFG motif adopts a specific orientation. In that orientation Phe595 rotates away from the αC-helix, allowing the formation of the Lys483 – Glu501 salt-bridge. Results indicate a correlation between the position of the αC-helix and the radius of gyration of the activation loop. In addition, B-Raf V600E exhibits a more flexible activation loop than wild type B-Raf. These mechanistic insights have implications for the development of new Raf inhibitors.

Biophysical and structural characterization of novel RAS-binding domains (RBDs) of PI3Kα and PI3Kγ

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Phosphatidylinositol-3-kinases (PI3Ks) are lipid kinases that phosphorylate phosphatidylinositol 4,5-bisphosphate to generate a key lipid second messenger, phosphatidylinositol 3,4,5-bisphosphate. PI3Kα and PI3Kγ require activation by RAS proteins to stimulate signaling pathways that control cellular growth, differentiation, motility and survival. Intriguingly, RAS binding to PI3K isoforms likely differ, as RAS mutations have been identified that discriminate between PI3Kα and PI3Kγ, consistent with low sequence homology (23%) between their RAS binding domains (RBDs). As disruption of the RAS/PI3Kα interaction reduces tumor growth in mice with RAS- and epidermal growth factor receptor driven skin and lung cancers, compounds that interfere with this key interaction may prove useful as anti-cancer agents. However, a structure of PI3Kα bound to RAS is lacking, limiting drug discovery efforts. Expression of full-length PI3K isoforms in insect cells has resulted in low yield and variable activity, limiting biophysical and structural studies of RAS/PI3K interactions. This led us to generate the first RBDs from PI3Kα and PI3Kγ that can be expressed at high yield in bacteria and bind to RAS with similar affinity to full-length PI3K. We also solved a 2.31 Å X-ray crystal structure of the PI3Kα-RBD, which aligns well to full-length PI3Kα. Structural differences between the PI3Kα and PI3Kγ RBDs are consistent with differences in thermal stability and may underly differential RAS recognition and RAS-mediated PI3K activation. While these high expression, functional PI3K RBDs will aid in interrogating RAS interactions and could aid in identifying inhibitors of this key interaction, further investigation into how oncogenic KRAS mutants bind and activate full-length PI3Ka will allow us to connect meaningful data from studies with our PI3Ka-RBD.

Monoubiquitination of KRAS at Lysine104 and Lysine147 Modulates its Dynamics and Interaction with Partner Proteins

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KRAS, a 21 kD guanine nucleotide binding protein that functions as a molecular switch, plays a key role in regulating cellular growth. KRAS undergoes post-translational modification by monoubiquitination at various locations, including at lysine104 (K104) and lysine147 (K147). Previous studies have suggested that K104 stabilizes helix-2/helix-3 interactions and K147 is involved in nucleotide binding. However, the impact of monoubiquitination at these residues on the overall structure, dynamics, or function of KRAS is not fully understood. In this study, we examined KRAS monoubiquitination at these sites using data from extensive (12 μs aggregate time) molecular dynamics simulations complemented by nuclear magnetic resonance spectroscopy data. We found that ubiquitin forms dynamic non-specific interactions with various regions of KRAS, and that ubiquitination at both sites modulates conformational fluctuations. Importantly, while ubiquitin modification at both K104 and K147 samples a broad range of conformations and conformational space around KRAS and does not form long-lasting non-covalent contacts, it adopts several preferred orientations relative to KRAS. To examine the functional impact of these preferred orientations, we performed a systematic comparison of the dominant configurations of the ubiquitin/KRAS simulated complex with experimental structures of KRAS bound to regulatory and effector proteins as well as a model membrane. Results from these analyses suggest that conformational selection and population shift may minimize the deleterious effects of KRAS ubiquitination at K104 and K147 on binding to some but not all interaction partners. Our findings thus provide new insights into the steric effects of ubiquitin and suggest a potential avenue for therapeutic targeting.

Thermodynamic and kinetic aspects of K-Ras4B activation/inactivation

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Using long-time molecular dynamics (MD) simulations, we probe the detailed atomistic behaviour of GTP-bound K-Ras4B proteins solvated with explicit water molecules. Here, rather than using only a few long trajectories, we use sets of many relatively short MD simulations, initiated from several initial structures relevant to the activation-inactivation mechanism, in an attempt to extract a detailed description of both the thermodynamic and equilibrium kinetic behaviour of wildtype K-Ras4B. We analyse first the detailed energy landscape built using two important distances, labelled d1 and d2 (i.e., distances between the beta-P atom of GTP and two key residues, T35 and G60), which were shown to be useful reaction coordinates in previous studies. Further, we estimate both populations and transition rates between the active and inactive states unveiled by this study, which suggest a more complex equilibrium conformational landscape than previously reported. Finally, using the detailed data on the wildtype behaviour, we obtain and report similarly detailed information of carefully chosen mutations. In particular, we show that a minimal mutation on the Switch 1 loop can lead to dramatic changes in both kinetic and thermodynamic properties of the protein.

Clustering RAS structures from the Protein Data Bank expands the landscape of characterized conformations.

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Mutations that shift the conformational equilibrium of RAS GTPases (KRAS, NRAS, and HRAS) towards the active state cause a variety of disorders, including 10-30% of human cancers. Attempts have been made to classify and study the conformations of wild-type (WT) and mutated RAS structures from the Protein Data Bank (PDB) using the principal component analysis (PCA) algorithm. However, there is no automated classification system that covers the majority of currently available structures or that is applicable to new ones as they are solved. We have developed a classification of RAS conformations utilizing the Density-Based Clustering of Applications with Noise (DBSCAN) algorithm with a distance metric that is a function of the backbone and side-chain dihedral angle values across residues of switches 1 and 2, which are the most conformationally dynamic loops on RAS. We analyzed 652 human KRAS, HRAS, and NRAS structures from the PDB and identified seven known and eleven novel RAS conformations. The known RAS conformations include four GTP-conformers, one GDP-conformer, and two nucleotide-free conformers. Several of the novel conformations are only found in the PDB with different classes of RAS inhibitors. In addition, one novel and three known GTP-conformers as well as one novel GDP-conformer are associated with RAS homodimer formation, a suggested prerequisite interaction for RAS binding to some effectors. Our conformational classification of RAS structures from the PDB will be made available in an open access webserver called RasCore to assist in structural study and comparison of WT and mutated forms of RAS proteins.

Structural Mechanism of PI3K Activation

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PI3Kα (PIK3CA), the second highly mutated oncogene in PI3K/Akt/mTOR pathway, is activated by the receptor tyrosine kinases (RTKs) and Ras on the membrane for cell growth and proliferation. PI3Kα is an obligate dimer consisting of p110α catalytic subunit and p85α regulatory subunit. The activation of PI3Kα features two events, i) the release of nH2 by phosphorylated tyrosine (pY) motifs in the RTK and ii) the membrane localization by Ras. However, given that the nSH2 domain is far from the catalytic site in kinase domain, the mechanism of PI3Kα activation by nSH2 release has been unclear. Our results suggest, upon nSH2 release, that PI3Kα undergoes the significant conformational change for activation. The iSH2 domain moves away from kinase domain. The activation loop (a-loop) becomes more flexible with more basic residue exposed for catalysis and membrane interactions. The analysis of the available PI3K structures are consistent with the simulations, confirming two structural features that distinguish the inactive and active PI3K conformations. The inactive PI3Ks have the “collapsed” a-loop and “IN” kα11, and the a-loop and kα11 in active PI3Ks are “extended” and “OUT”. These insights provide the structural and functional explanations for the hotspot and weak oncogenic mutations in PI3Kα and outline the PI3K allosteric drug discovery principle.
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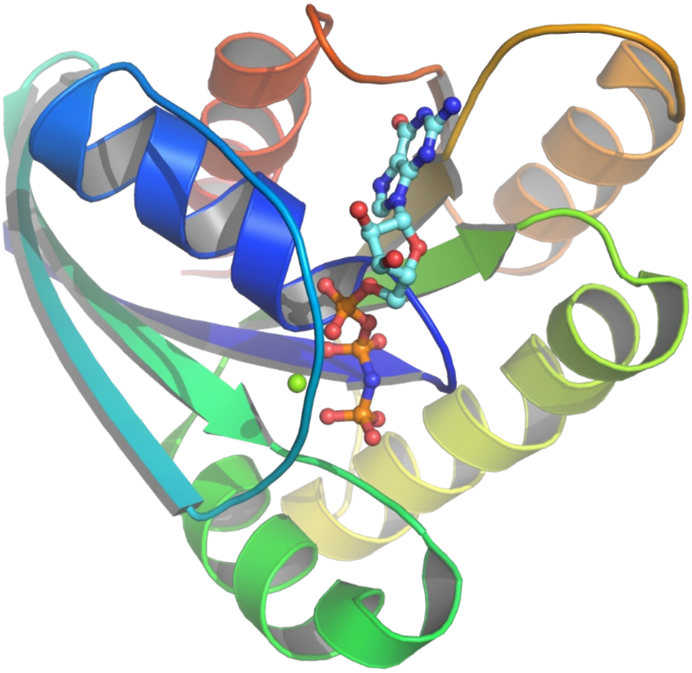

RASopathies

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Neurofibromin/NF1 is Not Just a Ras GAP But Also a Co-repressor for Estrogen Receptor-α Transcriptional Activity

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Germline loss of *NF1* causes *n*euro*f*ibromatosis type-*1*. *NF1* is also a key tumor suppressor gene lost somatically in cancer. *NF1* encodes neurofibromin, best known as a GAP (GTPase Activating Protein) that inhibits Ras signaling. However, *NF1* missense mutations affecting just the GAP domain are rare in primary cancer.

Up to 80% of breast cancers are driven by estrogen receptor-α (ER), which can be inhibited by endocrine therapy. However, the most deaths from breast cancer still come from the ER^+ subset, due primarily to drug resistance. In a previous tumor exosome sequencing study, we found that while no GAP mutations were found in our cohort, death and relapse after monotherapy with tamoxifen (an ER antagonist) strongly correlated with *NF1* nonsense/frameshift mutations (hazard ratio ≈ 3). *NF1* mutations were also enriched in metastases. Consistent with nonsense mRNA decay, *NF1* mRNA levels were greatly reduced in tumors carrying a nonsense/frameshift mutation, and low *NF1* mRNA levels also correlated with resistance to aromatase inhibitors (AIs), which lower estradiol levels. These data suggest that loss of not just the GAP domain but also additional functional domains in NF1 is necessary to promote aggressive tumor activities.

We now report that neurofibromin is also a direct ER transcriptional co-repressor containing two leucine/isoleucine-rich motifs, which are frequently mutated in cancer. Disrupting ER-binding does not affect GAP activity; conversely, inactivating the GAP activity does not impact ER-binding. Consequently, neurofibromin-depletion enhances ER-dependent gene expression causing estradiol hypersensitivity and tamoxifen agonism, explaining the poor prognosis associated with *NF1*-loss in tamoxifen and AI-treated ER^+ breast cancer. Neurofibromin-deficient ER^+ breast cancer cells initially retain sensitivity to selective estrogen receptor degraders (SERDs, e.g., fulvestrant). However, Ras activation does play a role in acquired SERD-resistance, which can be reversed upon MEK inhibitor (MEKi) addition, and SERD-MEKi combinations induce tumor regression.

Thus, neurofibromin is a dual repressor for both Ras and ER signaling. Neurofibromin depletion marks a distinct breast cancer subset in which the choice of endocrine treatment may be critical, and where SERD-MEKi combinations may deserve further clinical investigation. ER repression by NF1 may also explain the sexually dimorphic characteristics of neurofibromatosis, where tumorigenesis is promoted by female puberty.

SPRED1 regulates Ras activity through retromer-dependent endosomal trafficking

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Germline mutations in genes that encode integral members or regulatory proteins of the Ras/MAPK pathway are causative of developmental disorders classified as RASopathies. SPRED1 (Sprouty-related EVH1 domain-containing protein 1) has been described as such a RASopathy gene causing the Legius syndrome. Despite the fact that SPRED1 has been reported to interact with the Ras-GAP neurofibromin (NF1), its precise functional principle has not been unraveled yet.

In this study, we investigate the molecular mechanism underlying the modulation of the Ras/MAPK pathway by SPRED1. We observed that overexpression of SPRED1 resulted in formation of abnormally large fragmented endosomes, causing a redistribution of Ras from the plasma membrane to the endosomal compartment. Transmission electron microscopy of SPRED1-overexpresssing cells highlighted numerous vesicle buds sprouting from the giant endosomes indicating that SPRED1 regulated fusion/fission processes. A proximity biotinylation screen followed by liquid chromatography-mass spectrometry-based quantitative proteomics was applied to identify SPRED1 interacting/proximal proteins. The screen revealed two proteins, DNM2 (dynamin-2) and SNX3, a component of endosomal retromer, that are involved in endosomal trafficking. Endogenous SNX3 accumulated on SPRED1-induced large endosomes confirming a potential link between the ability of SPRED1 to induce giant vesicle formation and its interaction with SNX3.

Our findings suggest that Spred1 is involved in Ras activity control by affecting its subcellular distribution during endosomal trafficking. We hypothesize that SPRED1 regulates post-endosomal retromer-dependent Ras-recycling to the plasma membrane, thus impairing downstream signaling along the Ras/MAPK pathway.

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The RIT1 C-terminus associates with lipid bilayers via charge complementarity

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RIT1 is a member of the Ras superfamily of small GTPases involved in regulation of cellular signaling. Mutations to RIT1 are involved in cancer and developmental disorders. Like many Ras subfamily members, RIT1 is localized to the plasma membrane. However, RIT1 lacks the C-terminal prenylation that helps many other subfamily members adhere to cellular membranes. We used molecular dynamics simulations to examine the mechanisms by which the C-terminal peptide (CTP) of RIT1 associates with lipid bilayers. We show that the CTP is unstructured and that its membrane interactions depend on lipid composition. While a 12-residue region of the CTP binds strongly to anionic bilayers containing phosphatidylserine lipids, the CTP termini fray from the membrane allowing for accommodation of the RIT1 globular domain at the membrane-water interface.

Advocating for Research on RASopathies: Genetic Disorders of the RAS Signaling Pathway

Lisa Schoyer, MFA, Beth Stronach, PhD; RASopathies Network https://RASopathiesNet.org

RASopathies Network (RASNet) is a nonprofit advocacy organization run by parents of children with neurodevelopmental syndromes caused by germline mutations in components of the Ras-MAPK signaling pathway. These syndromes are collectively referred to as 'RASopathies' and include: Costello syndrome (CS) caused by mutations in HRAS; Cardio-facio-cutaneous (CFC) syndrome with mutations in BRAF, MAP2K1/2, or KRAS; Neurofibromatosis type 1 (NF1) caused by neurofibromin RAS-GAP mutations; Legius syndrome with SPRED1 mutations; and Noonan and Noonan-like syndromes (NS, NSML, NS-LAH) linked to >18 genes, with PTPN11 (Shp2) mutations being the most prevalent. The vast majority of RASopathy mutations result in activation of signaling. Excess signaling during development causes clinical manifestations including distinct facial features, feeding difficulties, growth delay, heart defects, sparse/coarse hair, cutaneous marks, short stature, and elevated cancer risk. RASopathies affect nearly every physiological system to various extents impacting quality of life and sometimes mortality.

RASNet provides support and information to families diagnosed with a RASopathy, raises awareness of the syndromes, and connects caregivers, researchers, and doctors, with the ultimate goal to accelerate research toward effective treatments and better quality of life. In particular, RASNet 1) convenes biennial symposia with clinicians, researchers, and RASopathy families, to discuss the current state of the field and bring attention to issues raised by patients; 2) engages stakeholders through partnerships, presentations, website and social media networks to build knowledge and community around the RASopathies; 3) encourages participation in research studies and; 4) raises funds for research projects; and 5) lays the groundwork for clinical trial participation. It is the hope of the RASopathies community that advances in the treatment of RAS-driven cancers will come to benefit individuals with RASopathies.

We gratefully acknowledge the RASopathies community, RASNet Board Members: Lisa Schill, Lee Johnson, Elisabeth Parker, Bruce Deckman, Cara Borian; Scientific Advisory Board and Advocate Advisory Board members; Intramural partners: Brigitte Widemann, Andrea Gross, Marielle Yohe, Pediatric Oncology Branch, CCR, NCI; and Mentor: William Timmer, NCI.

The novel role of Ras in cell competition and cancer

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Cell competition could be tumor-suppressive or tumor-promoting, depending on whether mutant cells lose or win. However, since loser and winner cells are not readily distinguishable within conventional animal models, cell competition is often “hidden” and thus under-studied. In *Drosophila*, genetic mosaic system is the enabling tool, in which mutant and wildtype (WT) cells coexist in the same animal and are labeled with distinct colors. Our lab developed a genetic system termed Mosaic Analysis of Double Markers (MADM) to address this problem in mice. Starting from a non-labeled mouse heterozygous for a tumor suppressor gene (TSG), MADM generates GFP-labeled TSG-null cells and RFP-labeled sibling WT cells. The ratio of green to red cell numbers (G/R ratio) reveals mutant cell over-expansion. Using MADM to inactivate *p53* and *NF1*, two commonly mutated TSGs in glioma patients, we pinpointed the oligodendrocyte precursor cell (OPC) as a cell-of-origin based on its massive G/R ratio (>100) long before malignancy. Surprisingly, overall OPC density in mutant brains barely increased in comparison to WT brains, revealing that mutant OPCs outcompete WT OPCs during their expansion. Furthermore, we demonstrated that the loss of *NF1* is sufficient for OPC competition, pinpointing the role of the over-activated Ras pathway. Most importantly, when we genetically equalized the fitness between WT and mutant OPCs by inactivating *NF1* in all OPCs, cell competition was blocked and gliomagenesis was prevented. Our studies not only revealed a novel role of Ras in cell competition, but also inspire one to consider unorthodox “equalization” strategies in cancer treatment.

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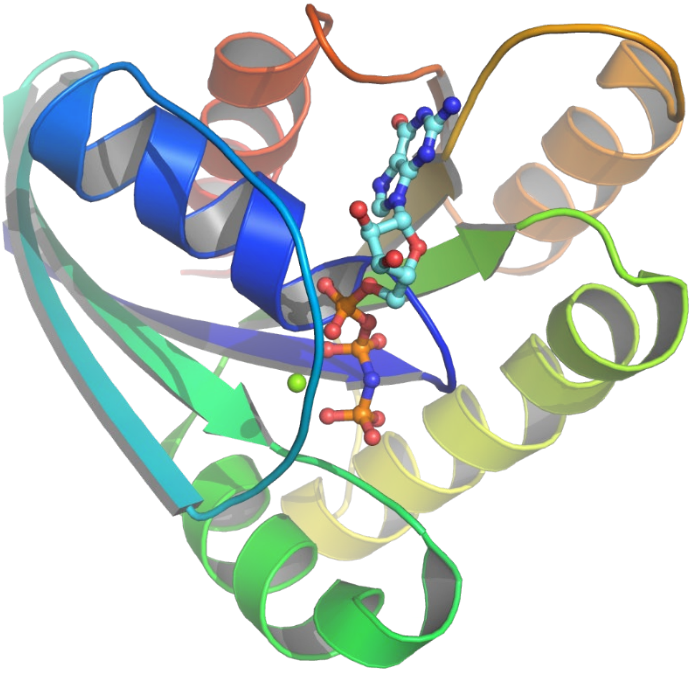

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Phase I clinical trial of trametinib and ponatinib in patients with non-small cell lung cancer harboring *KRAS* mutations

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Background: Somatic *KRAS* mutations occur in 25% of patients with metastatic NSCLC. There are currently no FDA approved targeted therapies for *KRAS* mutant NSCLC. MEK inhibitors have been tested in clinical trials with poor efficacy, likely due to adaptive resistance mechanisms leading to rebound in MAPK signaling. Compensatory activation of FGFR1 was identified as potential critical mechanisms of trametinib resistance in *KRAS* mutant lung cancer models, and combination therapy with trametinib and ponatinib was synergistic leading to tumor shrinkage in *in vivo* and *in vitro*. We conducted a phase I study to assess the safety and efficacy of the combination of trametinib and ponatinib in patients with *KRAS* mutant advanced NSCLC.

Methods: Activating *KRAS* mutations were identified by NGS testing in patients with advanced NSCLC. Patients were required to have prior treatment with chemotherapy and immune checkpoint inhibitor. Standard 3+3 dose escalation was employed. Toxicities were assessed according to National Cancer Institute Common Terminology Criteria v4.1. Disease assessments were performed every 2 cycles and response was assessed by RECIST 1.1 criteria. Patients were treated with study therapy until intolerable toxicity or progression of disease.

Results: 12 patients with *KRAS* mutant NSCLC were treated with trametinib/ponatinib. G12C mutations were identified in 50% (n=6) patients, other subtypes enrolled included G12D, G12C, G12V, and Q61H. A total of 6 patients were treated at dose level 1 (trametinib 2mg/ponatinib 15mg) and 6 patients were treated dose level 2 (trametinib 2mg/ponatinib 30mg). The most common toxicities observed were rash, diarrhea, and fever. Serious adverse events were reported in 5 patients including one death on study, and 4 additional cardiovascular events. 9 of 12 patients were evaluable for response, no confirmed partial responses were observed. The median duration of time on study was 43 days.

Conclusion: In this phase I dose escalation clinical trial, treatment with trametinib and ponatinib was associated with risk of cardiovascular and bleeding toxicities in patients with *KRAS* mutant advanced NSCLC. While 5 of 9 patients with evaluable disease were observed to have modest disease shrinkage while on treatment, responses were not durable. Exploring combination MEK and FGFR1 inhibition in future studies is warranted given preclinical studies and activity observed, but alternative agents should be considered to improve safety and tolerability.

Assessing the NRAS 5’ UTR as a Target for Small Molecules

Sumirtha Balaratnam*, Zachary Torrey, Dave Calabrese, and John S. Schneekloth Jr.

Neuroblastoma RAS (NRAS) is an oncogene that is deregulated and highly mutated in many Melanomas and Acute Myeloid Leukemias. Constitutively activated NRAS induces the MAPK and AKT signaling pathways and leads uncontrolled proliferation and cell growth, making it an attractive target for small molecule inhibition. However, like all RAS-family proteins, it has proven difficult to identify molecules that inhibit the protein. An alternative approach would involve targeting the NRAS mRNA. The 5’ untranslated region (5’UTR) of the NRAS mRNA is reported to contain a non-canonical secondary structure G-quadruplex (GQ), that regulates the translation process of NRAS mRNA. Stabilizing the GQ structure in NRAS by small molecules provides an alternative approach to reduce NRAS protein expression in cancer cells. Here we present a novel class of small molecule that bind to the G-quadruplex located 5’UTR of the NRAS mRNA. We used a small molecule microarray screen to identify molecules that selectively bind to the NRAS GQ. Biophysical studies, including thermal melt, fluorescence titration and SPR analysis, demonstrated that the compound binds reversibly to the NRAS GQ structure with nanomolar affinity. A Luciferase based reporter assay indicated that one compound inhibits the translation of NRAS via stabilizing the NRAS-GQ *in vitro.* Structure probing and sequencing analysis provided further insights into the structure and targetability of the 5’ UTR.

Overcoming adaptive resistance to KRAS and MEK inhibitors by co-targeting mTORC1/2 complexes in pancreatic cancer

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Activating KRAS mutations are found in over 90% of pancreatic ductal adenocarcinomas (PDAC), yet KRAS has remained a difficult target to inhibit pharmacologically. While mutant KRAS-specific inhibitors have recently been developed, there is increasing awareness that adaptive resistance to these inhibitors limits their efficacy as single agents. Here, we demonstrate, using several human and mouse models of PDAC, rapid acquisition of tumor resistance in response to genetic or pharmacologic targeting of KRAS or MEK, associated with increased phosphorylation of Akt and Rictor, in an ILK-dependent manner. While pharmacologic inhibition of mTORC1/2 results in a compensatory increase in ERK phosphorylation, combinatorial treatment of PDAC cells with either KRAS or MEK inhibitors together with mTORC1/2 inhibitors results in synergistic cytotoxicity and cell death reflected by sustained inhibition of p-ERK and p-Rictor/p-Akt, and of downstream regulators of protein synthesis and cell survival. Importantly, relative to single agents alone, this combination leads to significant inhibition of tumor growth *in vivo*, including durable tumor regressions and increased survival. Interestingly, treatment with single agents alone leads to the dramatic induction of metastatic progression that was inhibited by the combination therapy. We have identified a highly effective combinatorial treatment strategy, using clinically viable inhibitors, which can be applied to PDAC tumors with different KRAS mutations.

Engineering subtilisin proteases that specifically degrade active RAS

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We describe the design, kinetic properties, and structures of engineered subtilisin proteases that degrade the active form of RAS by cleaving a conserved sequence in switch 2. RAS is a signaling protein that, when mutated, drives a third of human cancers. To generate high specificity for the RAS target sequence, the active site was modified to be dependent on a cofactor (imidazole or nitrite) and protease sub-sites were engineered to create a linkage between substrate and cofactor binding. Selective proteolysis of active RAS arises from a 2-step process wherein sub-site interactions promote productive binding of the cofactor, enabling cleavage. Proteases engineered in this way specifically cleave active RAS *in vitro*, deplete the level of RAS in a bacterial reporter system, and also degrade RAS in human cell culture. Although these proteases target active RAS, the underlying design principles are fundamental and will be adaptable to many target proteins.

Ras inhibitors and immune checkpoint therapy for pancreatic cancer

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Pancreatic cancer remains a lethal disease with no highly effective treatment options. Immune checkpoint inhibitors have recently produced some complete responses in pancreatic cancer patients, but these were rare events. Pancreatic cancers are almost invariably driven by RAS mutations. One of the actions of mutant RAS is to promote an immunosuppressive tumor microenvironment, which may contribute to the poor performance of checkpoint inhibitors in this disease. Recent success in the development of targeted inhibitors of RAS have raised hopes that they may be applied to pancreatic cancer. It has been speculated that they might enhance the action of immune checkpoint inhibitors.

We have developed a pan-RAS inhibiting small molecule that is active against pancreatic tumor systems in vivo. We have recently tested it against a syngeneic model of RAS driven pancreatic cancer in combination with an anti-PDL1 antibody. The combination treatment exhibited enhanced anti-tumor activity. This provides proof of principal for the use of the imminent RAS inhibitors with established immunomodulatory therapies.

Biophysical, cellular, and *in silico* characterization of the pan-RAS inhibitor ADT 007

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Mutations in the three *RAS* oncogenes, which occur in approximately 30% of all human cancers, drive tumor growth and metastasis by aberrant activation of RAS signaling. Pan-RAS inhibitors can potentially target all RAS isozymes co-expressed in tumors to block RAS activation. A novel class of pan-RAS inhibitors was identified by screening a focused library of indenes using a phenotypic assay involving cancer cells with high (mutant RAS) vs. low (mutant RAF) levels of activated RAS-GTP. ADT 007 emerged following extensive chemical optimization to improve potency and selectivity. ADT 007 supressed RAS-driven 2D and 3D cancer cell growth, pan-RAS activation, and RAS signaling with low nM IC_{50} potency values in a panel of cancer cell lines harboring various RAS mutations or isozymes. Protein NMR (HSQC) using ADT 007 and ^{15}N-enriched wild-type KRAS4B identified multiple chemical shift changes and signal broadenings, including those corresponding to amino acids in the nucleotide binding pocket of KRAS. Binding was only observed for GDP-loaded Mg^{2+}-chelated KRAS. Similarly, nucleotide exchange experiments using a fluorescent GTP analog revealed that ADT 007 treatment decreased the rate of nucleotide binding only when accompanied by a Mg^{2+} chelation step. An unbiased pharmacotranscriptomic analysis of ADT 007-sensitive and resistant cancer cell lines revealed a molecular signature of compound sensitivity that included overexpression of the RAS family protein, RAB-3B. Uncovering a more complete understanding of ADT 007's mechanism of RAS binding may reveal new vulnerabilities for the treatment of RAS-driven malignancies.

Characterization of autophagy inhibition as a therapeutic strategy for pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is characterized by KRAS- and autophagy-dependent growth. Autophagy is a multi-step, lysosomal-mediated process whereby cells degrade and recycle macromolecules to sustain growth. We and others recently demonstrated that inhibition of the RAF-MEK-ERK pathway resulted in upregulated autophagic flux, and that dual treatment with the autophagy inhibitors hydroxychloroquine (HCQ)/chloroquine (CQ) and inhibitors of ERK and MEK synergistically blocked PDAC growth. Currently, HCQ/CQ are the only clinically approved autophagy inhibitors; however, it is limited by low potency and lack of specificity. To identify genes that when lost enhance the anti-proliferative effect of HCQ treatment, we performed a CRISPR-Cas9-mediated genetic loss-of-function screen in the presence of CQ. Interestingly, we identified multiple genes that encode proteins that function in all key stages in the autophagic pathway. Preliminary results determined that treatment of PDAC cells with anti-autophagy combinations targeting multiple nodes in the pathway, or ‘vertical inhibition’ of the pathway, resulted in further reduction of autophagic flux relative to inhibition of any single node and also synergistically reduced PDAC cell proliferation. Furthermore, combining ERK inhibition with vertical inhibition of the autophagy pathway synergistically reduced cell growth in a panel of PDAC cell lines. Ongoing studies are delineating the mechanism underlying the synergy observed with anti-autophagy inhibitor combinations and further characterization of this strategy in additional preclinical models of PDAC. These studies will enhance our understanding of autophagy and aid in the development of novel combination therapies to target autophagy for PDAC treatment.

Combination screening of KRAS^{G12C} specific inhibitors with other targeted therapies in patient-derived multi-cell type multicellular tumor spheroids

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Covalent inhibitors targeting a specific KRAS^{G12C} (Gly12 to Cys12) variant were recently developed and demonstrate encouraging clinical activity in patients with tumors driven by the variant protein. We investigated the growth inhibitory activity of two KRAS^{G12C} variant-specific inhibitors, AMG-510 and MRTX-1257, in combination with other targeted therapies. Twenty patient-derived cancer cell lines available from the NCI (https://pdmr.cancer.gov/models/database.htm), which were characterized for their KRAS status and related targets, were grown as multicellular 3D complex spheroids. The complex spheroids, incorporating tumor cells, endothelial cells, and mesenchymal stem cells, were grown for three days prior to drug exposure. As single agents, AMG-510 and MRTX-1257 showed selectivity for inhibiting cancer cell lines containing the KRAS^{G12C} variant. The pancreatic cell line 323965-272-R-J2, carrying a KRAS^{G12C} variant, exhibited an enhanced sensitivity to the combination of the allosteric mTOR inhibitor everolimus and MRTX-1257, while no such effect was detected in pancreatic cell lines with wildtype or other KRAS variants. Similarly, a combination of the mTOR kinase inhibitor sapanisertib with MRTX-1257 substantially reduced the cell viability of all three cell lines expressing KRAS^{G12C}. In the KRAS^{G12C} variant melanoma cell line 299254-011-R-J1, a combination of the FGFR inhibitor erdafitinib and either KRAS^{G12C} inhibitor produced greater than additive cell killing. Lastly, combinations of either KRAS^{G12C} inhibitor with abemaciclib induced greater than additive cytotoxicity in the pancreatic cancer cell line 323965-272-R-J2. These preclinical results provide guidance for the selection of combination regimens with KRAS^{G12C} inhibitors that might improve clinical efficacy.

Defining the KRAS-regulated kinome in KRAS-mutant pancreatic cancer

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Oncogenic KRAS drives cancer growth by activating diverse signaling networks, not all of which have been fully delineated. We set out to establish a system-wide profile of the KRAS-regulated kinase signaling network (kinome) in KRAS-mutant pancreatic ductal adenocarcinoma (PDAC). We knocked down KRAS expression in a panel of six cell lines, and then applied Multiplexed Inhibitor Bead/Mass Spectrometry (MIB/MS) chemical proteomics to monitor changes in kinase activity and/or expression. We hypothesized that depletion of KRAS would result in downregulation of kinases required for KRAS-mediated transforming activities, and in upregulation of other kinases that could potentially compensate for the deleterious consequences of the loss of KRAS. We identified 15 upregulated and 13 downregulated kinases in common across the panel. In agreement with our hypothesis, all 15 of the upregulated kinases have established roles as cancer drivers (e.g., SRC, TGFBR1, ILK), and pharmacologic inhibition of the upregulated kinase, DDR1, suppressed PDAC growth. Interestingly, 11 of the 13 downregulated kinases have established driver roles in cell cycle progression, particularly in mitosis (e.g., WEE1, Aurora A, PLK1). Consistent with a crucial role for the downregulated kinases in promoting KRAS-driven proliferation, we found that pharmacologic inhibition of WEE1 also suppressed PDAC growth. The unexpected paradoxical activation of ERK upon WEE1 inhibition led us to inhibit both WEE1 and ERK concurrently, which caused further potent growth suppression and enhanced apoptotic death than WEE1 inhibition alone. We conclude that system-wide delineation of the KRAS-regulated kinome can identify potential therapeutic targets for KRAS-mutant pancreatic cancer.

Targeting mitochondrial function as a therapeutic strategy for KRAS-mutant pancreatic cancer

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Although the genetic landscape of pancreatic ductal adenocarcinoma (PDAC) is now well-defined, no clinically effective targeted therapies exist for PDAC. The KRAS oncoprotein, mutationally activated in ~95% of PDAC, orchestrates multiple metabolic changes to support the elevated energy demands of pancreatic cancer cells. Thus, targeting PDAC metabolism is a promising therapeutic avenue. Our studies take two complementary approaches towards this effort that are centered on mitochondrial function. The KRAS and its major effector signaling pathway, the RAF-MEK-ERK MAPK cascade, activates DRP1 to drive mitochondrial fragmentation, whereas OPA1 and MFN1 promote mitochondrial fusion. To address the role of mitochondrial morphology and metabolism in PDAC, we suppressed DRP1, which increased mitochondrial fusion, impaired cell growth, and caused a compensatory increase in autophagy. Conversely, suppression of OPA1 and MFN1 increased mitochondrial fragmentation but did not enhance cell growth. These results suggest that fragmented mitochondria are necessary but not sufficient to support KRAS-dependent PDAC growth. Future studies include RPPA profiling to understand the signaling changes, as well as examining the effects on cellular metabolism conferred by mitochondrial dynamics in PDAC. As a second approach, we evaluated ONC201, a clinical candidate agonist of the mitochondrial protease ClpP, that causes proteolytic degradation of mitochondrial proteins. We found that ONC201 inhibited PDAC cell growth alone and in combination with the ERK-selective inhibitor SCH772984. Our ongoing studies are evaluating the consequences of ONC201 on mitochondrial morphology and metabolism, as well as on other cellular metabolic activities. In summary, our results support a therapeutic value in targeting mitochondrial dysfunction.

The integrated stress response exposes a therapeutic vulnerability in KRAS driven lung cancer

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Mutations in KRAS account for ~25% of lung cancer cases and are refractory to therapy. Tumors with KRAS mutations encounter increased levels of genotoxic, proteotoxic and metabolic stress, which disrupt proliferation and homeostasis. Tumor growth requires induction of pathways to promote adaptation to stress. The Integrated Stress Response (ISR) plays a key role in adaptation to stress via phosphorylation of eukaryotic initiation factor 2 (p-eIF2). When ISR is activated, p-eIF2 mediates a translational-transcriptional reprogramming of genes with roles in survival and adaptation.

We demonstrate that ISR is pivotal in the development of lung adenocarcinoma. Specifically, analysis of 928 lung adenocarcinoma patients showed that increased p-eIF2 associates with a significant decrease in overall survival by ~12 months. These findings underscore the prognostic significance of ISR in the development and treatment of human lung adenocarcinoma.

We investigated the role of ISR in mouse models of KRAS driven-lung cancer by genetic and pharmacological means. We demonstrate the pro-tumorigenic function of ISR via its ability to stimulate phosphorylation of mitogen activated kinase ERK. Mechanistically, we show that increased p-eIF2 translationally represses dual specificity phosphatase DUSP6, which antagonizes ERK phosphorylation.

Our work also demonstrates that pharmacological ISR inactivation is highly effective for treatment of KRAS lung cancer in mice. That is, ISR disruption by inhibiting the eIF2α kinase PERK or inactivating the translational effects of p-eIF2 by the integrated stress response inhibitor (ISRIB) substantially reduces lung tumor growth in mice.

Our findings provide a novel rationale for implementing ISR-based regimens in lung adenocarcinoma treatment.

Combination therapies with CDK4/6 inhibitors to treat *KRAS*-mutant pancreatic cancer

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The development of targeted therapies for pancreatic ductal adenocarcinoma (PDAC) is a significant unmet clinical need. Two frequent genetic events in PDAC, mutational activation of *KRAS* and loss of the tumor suppressor *CDKN2A*, converge on activation of the cell cycle regulatory kinases CDK4 and CDK6. However, single agent therapy targeting either the KRAS RAF-MEK-ERK effector signaling pathway (with MEK inhibition) or CDK4/6 alone have not shown clinical efficacy in PDAC. We found that concurrent CDK4/6 inhibitor (CDK4/6i) and ERK MAPK inhibitor (ERKi) treatment synergistically blocked PDAC cell line and organoid growth by blockade of CDK4/6i-induced compensatory signaling changes and by induced synergistic alteration of cellular signaling. Based on these findings, we initiated a Phase I clinical trial evaluating the ERKi ulixertinib in combination with the CDK4/6i palbociclib in patients with advanced PDAC (NCT03454035). To search for additional combinations, we applied a CRISPR-Cas9 loss-of-function screen and identified a spectrum of functionally diverse genes whose loss enhanced CDK4/6i growth inhibitory activity. These genes were particularly enriched around diverse signaling nodes including PI3K-AKT-mTOR signaling, cell cycle regulatory proteins particularly centered on CDK2 activation, SRC family kinases, HDAC proteins, autophagy-activating pathways, chromosome regulation and maintenance, and DNA damage & repair pathways. Novel combinations were validated using siRNA and small molecule inhibitor-based approaches. We also identified genes whose loss imparted a survival advantage (e.g., *RB1, PTEN, FBXW7*), suggesting possible resistance mechanisms to CDK4/6 inhibition. In summary, we identified novel combinations with CDK4/6i that may benefit PDAC patients clinically.

Discovery of potent SOS1 inhibitors and their pharmacological investigations to disrupt the RAS-SOS1 interaction

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Mutants of *RAS* are major oncogenes and are prevalent in many human cancers, however efforts to develop drugs that directly inhibit the corresponding constitutively active RAS proteins have so far been successful only for KRas-G12C. We focused on SOS1, the guanine nucleotide exchange factor (GEF), and an activator of RAS. We identified good starting points for medicinal chemistry activities using both high-throughput and fragment screens. Initial optimizations resulted in the discovery of the first nanomolar SOS1 inhibitors, which effectively downregulated active RAS in tumor cells.

Here, the key findings on our path to identifying novel potent and cellular active small molecule inhibitors will be described. These inhibitors efficiently disrupted the interaction between KRAS and its exchange factor SOS1, this mode of action was confirmed by a series of biophysical techniques. The binding sites, mode of action and selectivity were elucidated using crystal structures of KRAS^{G12C}–SOS1, SOS1 and SOS2. By preventing formation of the KRAS–SOS1 complex, these inhibitors block the reloading of KRAS with GTP and, therefore, showed antiproliferative activity. Our probe **BAY-293** selectively inhibited the KRAS–SOS1 interaction with an IC$_{50}$ of 21 nM and is a valuable chemical probe for further investigations. In cells with wild-type KRAS the complete inhibition of the RAS–RAF–MEK–ERK pathway was observed. In a mutant KRAS cell line, SOS1 inhibition resulted in a reduction of pERK activity by 50%. In vitro and in vivo combination experiments revealed strong synergy of BAY-293 with covalent inhibitors of KRAS G12C. Together, the data indicate that inhibition of GEFs may represent a new viable approach for targeting RAS-driven tumors.

Title: Characterisation of a cyclic peptide that binds the p110α-RBD and blocks its interaction with RAS

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p110α is a member of the PI3K family of kinases that functions downstream of RAS. RAS proteins contribute to the activation of p110α by directly interacting with its RAS binding domain (RBD), resulting in promotion of many cellular functions such as cell growth, proliferation and survival. Previously our lab has shown that blocking the interaction of p110α with oncogenic KRAS by introducing specific mutations in the RBD had a significant effect on tumour growth and initiation, highlighting the significance of the p110α/KRAS interaction in tumour progression and maintenance.

Here we report the discovery and characterisation of a cyclic peptide inhibitor (Cyclo-RBDα) that interacts with the p110α-RBD and blocks the interaction with KRAS. Cyclo-RBDα was discovered by using the “split-intein cyclisation of peptides and proteins” (SICLOPPS) technique, that allows the production and screening of cyclic peptides in bacteria. The primary cyclic peptide hit from the screen initially showed a weak affinity for the p110α-RBD (Kd about 380μM). However, after two rounds of amino acid substitution, this led to Cyclo-RBDα, with an improved affinity for p110α-RBD of about 1μM. Furthermore, we show that Cyclo-RBDα binds selectively to the p110α-RBD but not to KRAS or the structurally-related RAF-RBD. In addition, Cyclo-RBDα effectively blocked the p110α/KRAS interaction in a dose dependent manner, using biophysical using (MST (Microscale thermophoresis) and NMR (Nuclear Magnetic Resonance)) , biochemical and cellular assays. We also tested the Cyclo-RBDα in several cancer cell lines carrying an oncogenic KRAS mutation, and found a reduction of the phospho-AKT levels in the presence of Cyclo-RBDα. From the above results, we believe that Cyclo-RBDα represents a new type of p110α inhibitor that may be a possible starting point for the design of future drugs against cancers that are driven by oncogenic RAS proteins.

Targeting the KRAS α4-α5 allosteric interface inhibits pancreatic cancer tumorigenesis

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RAS is a small molecular weight GTPase and the most commonly mutated oncogene in cancer. Roughly 20% of all cancers harbor mutations in one of three RAS genes (H, K or NRAS) with pancreatic cancers harboring RAS mutations in >90% of tumors. Therapeutically targeting RAS has been challenging due to the lack of deep pockets for binding pharmacologic agents. However, the recent development of KRAS(G12C) inhibitors that have shown promising clinical efficacy, has brought new hope to the possibility of pharmacologically inhibiting RAS. While KRAS(G12C) is frequently expressed in lung cancers, it is rare in more aggressive RAS addicted tumors such as pancreatic ductal carcinoma (PDAC). Thus, there remains an unmet need to develop inhibitors that target the more prominent KRAS mutants. This need demands devising novel strategies to inhibit RAS. Previously, we described an innovative approach to identify vulnerabilities in RAS using Monobody technology and isolated the RAS inhibitory Monobody called NS1. The NS1 selectively inhibits signaling and oncogenic transformation by both H/KRAS. Furthermore, NS1 inhibits the growth of KRAS-mutant human lines in xenograft models. However, these tumor models lack a functional immune system. Here, we evaluated the efficacy of targeting the α4-α5 interface of KRAS as an approach to inhibit PDAC development using an immunocompetent orthotopic mouse model. Using pancreatic tumor cells derived from the KPC mouse, a widely used model for pancreatic cancer, we established a stable subclone in which NS1 expression was regulated by doxycycline (DOX) treatment. Although human and mouse KRAS differ by a single amino acid [Asp (D) to Glu (E)] at position 132 in the NS1 binding region of the allosteric lobe, NS1 bound similarly to human and mouse KRAS both *in vitro* and *in vivo*. Chemically regulated NS1 expression inhibited ERK and AKT activation in KRAS(G12D) mutant KPC PDAC cells and reduced the formation and progression of pancreatic tumors. NS1-expressing tumors were characterized by increased infiltration of CD4+ T helper cells. However, we did not observe a significant change in the infiltration of CD8+ cytotoxic T cells or macrophages. These results suggest that targeting the α4-α5 allosteric interface of KRAS may represent a viable therapeutic approach for inhibiting KRAS-mutant pancreatic tumors.

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Inhibition of valosin-containing protein (VCP/p97) induces autophagic flux and ERK MAPK signaling and enhances therapeutic efficacy of autophagy and ERK inhibitors in pancreatic cancer

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We and others have recently shown that ERK MAPK inhibitors render KRAS-mutant pancreatic ductal adenocarcinoma (PDAC) cells addicted to autophagy and sensitize them to autophagy inhibition, e.g., to chloroquine (CQ). CRISPR-Cas9 loss-of-function screens of the druggable genome identified genes involved in the DNA Damage Response (DDR) as significant sensitizers to ERK inhibitors and to CQ. To further evaluate the DDR pathways and to identify additional potential therapeutic targets for KRAS-mutant PDAC, we performed a focused CRISPR-Cas9 loss-of-function screen targeting genes involved in the DDR. This screen identified VCP/p97 as an essential gene in pancreatic cancer cell lines, and VCP loss sensitized PDAC cell lines to inhibition of autophagy and of ERK1/2. VCP is a member of the AAA, or extended family of ATPases associated with various cellular activities, ATPase family. It transports misfolded, ubiquitinated proteins for degradation and maintains protein homeostasis and genomic stability. VCP inhibition alone resulted in growth inhibition and induction of apoptosis in KRAS-mutant PDAC cell lines. Interestingly, VCP loss induced compensatory autophagic flux and ERK MAPK signaling. We therefore treated PDAC cells concurrently with inhibitors of VCP and of each process. Concurrent treatment using VCP inhibitor with CQ or with ERK inhibitor suppressed growth and enhanced apoptosis compared to each inhibitor alone. Finally, we tested the triple combination of VCP, ERK, and CQ, which further enhanced growth suppression and induced apoptosis. We show that targeting VCP may enhance the efficacy of autophagy and ERK inhibitors in treating PDAC.

LKB1 loss rewires stress signaling-induced apoptotic protein dynamics and sensitizes *KRAS*-mutant non-small cell lung cancers to combined MAPK + MCL-1 blockade

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There are currently no approved targeted therapies for *KRAS*-mutant non-small cell lung cancers (NSCLC). The development of mutant-specific covalent inhibitors of KRAS G12C has invigorated hope that clinically effective KRAS-targeted therapies are within reach. While these agents have shown activity in early phase clinical trials, identification of specific vulnerabilities conferred by common co-occurring mutations in *KRAS*-mutant NSCLC may enable development of combination therapies with enhanced activity in distinct subsets of patients. We screened a panel of *KRAS*-mutant NSCLC cell lines and observed that loss of *STK11/LKB1* is associated with increased sensitivity to combined MAPK (either MEK inhibitor or KRAS G12C inhibitor AMG 510) and MCL-1 inhibition (AMG 176). Restoration of LKB1 expression in *LKB1*-deficient cell lines and mouse xenograft tumors blunted the apoptotic response to MAPK + MCL-1 inhibition; conversely, deletion of *LKB1* in *LKB1* wild-type models restored the sensitivity. Mechanistically, *LKB1* deficiency is associated with an altered phosphoproteome. *LKB1* loss increased cellular stress leading to hyperactivation of JNK1, phosphorylation and stabilization of MCL-1 protein, and increased BIM sequestration by MCL-1. Upon suppression of MAPK signaling, *LKB1*-deficient cells exhibited greater levels of BIM bound to MCL-1 that could be liberated by AMG 176 to induce apoptosis. Ex vivo treatment of tumor tissue from a *KRAS-LKB1* mutant NSCLC patient with AMG 510 increased MCL-1 dependent priming. These results uncover a novel link between LKB1, cellular stress, and the regulation of MCL-1. Moreover, our study provides preclinical rationale for the exploration of combined KRAS G12C + MCL-1 inhibitors for *KRAS-LKB1* mutant patients.

Loss of protein phosphatase 6 confers resistance to MAP kinase pathway inhibition via NF-κB activation

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A major challenge for targeting the MAP kinase (MAPK) pathway using RAF, MEK and ERK inhibitors is intrinsic and acquired resistance in tumors with *RAS* and *RAF* mutations. To understand the mechanisms that could lead to resistance to MAPK pathway inhibitors, we performed a CRISPR knockout screen in *KRAS* mutant cancer cells treated with the MEK1/2 inhibitor trametinib and the ERK1/2 inhibitor SCH772984. We found that loss of the catalytic subunit of protein phosphatase 6, PPP6C, confers resistance to these compounds in *KRAS* mutant colorectal cancer cells. In addition, PPP6C loss also confers resistance to the BRAF inhibitor PLX4032 in *BRAF* mutant colorectal cancer and melanoma cells. Mechanistically, PP6 functions as a negative regulator of the TAK1–IKKβ–NF-κB pathway. PPP6C loss leads to the hyperactivation of NF-κB signaling. This preserves cyclin D1 expression and blunts G1 arrest induced by acute MAPK pathway inhibition. Pharmacological inhibition of IKKβ, as well as CRISPR-mediated knockout of *MAP3K7/TAK1* and *RELA/P65* genes restore PPP6C-deficient cancer cells' sensitivity to MAPK pathway inhibitors *in vitro* and in mouse xenograft models. *PPP6C* is a tumor suppressor gene that is mutated in approximately 1% of colorectal adenocarcinomas and 8% of cutaneous melanomas. A common hotspot mutation in PPP6C at amino acid R264 behaves as a loss-of-function mutant towards NF-κB signaling. Our findings identify the PP6–NF-κB axis as a mediator of resistance to MAPK pathway inhibitors and suggest the NF-κB pathway as a co-target in *PPP6C* mutant cells in this context.

Calmirasone1 – a covalent calmodulin inhibitory tool compound to study the cell biology of K-Ras-associated stemness

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Calmodulin (CaM) is involved in cell cycle regulation and present at higher concentrations in malignant tissues and cancer cells. It was therefore pursued as a cancer drug target in the 1980s. Current data suggest that Ca^{2+}/CaM acts as a trafficking chaperone for K-Ras4B (hereafter K-Ras), the highly mutated Ras isoform and the focus of current Ras drug development efforts. We previously identified the covalent CaM inhibitor ophiobolin A (OphA), a naturally occurring fungal sesterterpenoid as inhibitor of the K-Ras membrane organization. Interestingly, OphA potently blocks the growth of cancer stem cell enriched spheroids of KRAS-mutant cancer cell lines. However, the application of OphA is limited by its unspecific, high cellular toxicity and complicated chemical synthesis.

We have developed a less toxic, functional analogue of OphA for application in cell biological experiments. A small library of benzazulenones was synthesized in six steps from guaiazulene. Compounds were assessed for cellular toxicity, inhibition of spheroid formation and their in vitro affinity to CaM.

On target activity in cells was determined using newly developed Bioluminescence Resonance Energy Transfer (BRET) biosensors for Ras-nanoclustering and the K-Ras/ CaM interaction. A formyl aminobenzazulenone, here named Calmirasone1, was identified as a novel and potent covalent CaM inhibitor. It has a 4-fold higher affinity for CaM as compared to OphA and reacts at least 7-times faster in vitro. Calmirasone1 showed similar K-Ras selectivity as OphA in cellular BRET-assays, while reacting much faster. Importantly, Calmirasone1 displayed a 40 – 260-fold lower unspecific cell toxicity on *HRAS* mutant cells, while it reached almost 50% of the activity of novel K-RasG12C specific inhibitors in 3D spheroid assays.

Given that three distinct genes encode the same CaM protein, CaM biology is difficult to track by genetic means. **Calmirasone1** now allows to chemically knock-down CaM to further investigate the cancer cell biology of the K-Ras and CaM associated stemness.

Live cell target engagement reveals the distinct pharmacology of clinical MEKi on macromolecular RAF-KSR-MEK-14-3-3 complexes

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Current methods of drug design and lead optimization focus on understanding drug-receptor interactions (i.e. target engagement) using a variety of in *vitro* and biophysical approaches. Here, we address issues that are inherent in these strategies (i.e. relevance of inhibitors outside of a cellular context etc.) by making use of bioluminescence resonance energy transfer (BRET) technology called NanoBRET that reads out the competition in cells that occurs between a fluorescently conjugated drug (tracer) and drug of choice for a luciferase tagged target protein. We have extended this technology to measure drug binding affinities of MEK inhibitors (MEKi) to protein complexes in the RTK-RAS-MAPK pathway. Using this assay, we found that MEKi such as Trametinib have preferences for certain MEK-RAF-KSR-14-3-3 complexes over others giving rise to MEKi profiles that highlight preferred complexes. These profiles will be important to design the next generation of inhibitors where inhibitors can be chemically fine-tuned to target MEK protein-protein interactions that are important for efficacy.

Novel Irreversible KRAS^{G12C} Inhibitors Identified Using Computational Molecular Design

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After the discovery of the druggable allosteric binding site containing the G12C mutation, KRAS^{G12C} has been the focus of attention in oncology research. In this work, we are reporting a computationally empowered approach aimed at identifying novel and selective KRAS^{G12C} covalent inhibitors. The workflow involved initial enumeration of a virtual library of compounds tailored for the KRAS switch-II binding site. Tools such as pharmacophore modeling, docking, and relative free energy calculations were deployed for the prioritization of molecules with the most interesting profiles. One of the identified scaffolds showed the ability to react with G12C and inhibit KRAS^{G12C}. Analogues were prepared to establish structure–activity relationships, while molecular dynamics simulations and crystallization of the inhibitor–KRAS^{G12C} complex highlighted an unprecedented binding mode.

Therapeutic KRAS^{G12C} inhibition alleviates KRAS-driven immunosuppression

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Therapeutic KRAS^{G12C} inhibitors may substantially improve KRAS-mutant cancer patient care, considering the encouraging clinical data emerging from ongoing clinical trials. Since oncogene-targeting therapies often lead to resistance, the development of combinatorial therapies will be of utmost importance to achieve long-lasting tumour control. Given the known immunosuppressive role of KRAS, clinical trials investigating the combination of KRAS^{G12C} inhibitors with immune checkpoint blockade is currently underway for NSCLC patients.

Here, we show that KRAS^{G12C} inhibition using MRTX1257 alleviates KRAS-driven immune suppression, through a myriad of mechanisms including the augmentation of tumour cell-intrinsic interferon responses. Despite harbouring a large number of somatic mutations, orthotopic 3LL (KRAS^{G12C}□NRAS) tumours are able to evade anti-tumour immune responses. Combining multicolour flow cytometry, RNA sequencing and imaging mass cytometry, we observed increased T cell activation and infiltration after MRTX1257 treatment in addition to enhanced antigen presentation cells and gene sets, creating an immune permissive microenvironment in these lung tumours. Despite these profound changes, combination of KRAS^{G12C} inhibitors and anti-PD-(L)1 antibodies fail to show synergism in our model, in contrast with Amgen's published data using an immunogenic model of colorectal cancer.

This data suggests that a pre-existing anti-tumour immunity may be crucial for clinical responses to KRAS inhibitors and immune checkpoint blockade combinations.

Henceforth, ongoing clinical trials investigating this approach will likely only benefit a subset of patients and further research will be needed to examine novel therapeutic approaches and combinations for refractory patients.

Co-Altered Genetic Lesions in HRAS-, KRAS-, and NRAS-Mutant Tumors Across Multiple Cancer Lineages

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Background: The clinical development of farnesyltransferase inhibitors (FTI) as targeted therapy for *HRAS*-mutant tumors has demonstrated mixed responses dependent on cancer type. Co-occurring mutations may affect tumor response. For instance, *NF1* mutations confer resistance to HRAS inhibition by the FTI tipifarnib in thyroid cancer mouse models. To uncover cooperative genetic events specific to *HRAS*-mutant tumors, we characterized mutations across multiple cancer lineages and compared these to those present in *KRAS*- and *NRAS*-mutant tumors.

Methods: Targeted sequencing data from MSK-IMPACT cohort and Dana-Farber Cancer Institute cases in the AACR Genomic Evidence Neoplasia Information Exchange (GENIE, Version 9.0) database was used to investigate tumors enriched for *HRAS*-mutations within salivary, thyroid, bladder, head and neck (HNSCC), melanoma, prostate, and non-small cell lung (NSCLC) cancers. Co-mutations were grouped into MAP kinase or PI3K pathway alterations. Frequency of co-altered mutations were plotted based on cancer type and compared to *KRAS*- and *NRAS*-mutant tumors. Only canonical oncogenic RAS mutations were evaluated.

Results: 244 *HRAS*-mutant tumors were identified compared to 2516 KRAS and 701 NRAS-mutant tumors. Oncogenic HRAS mutations were found in 19/415 (4.6%) salivary, 45/1138 (4.0%) thyroid, 76/2028 (3.7%) bladder, 28/1056 (2.7%) head and neck, 34/2032 (1.7%) melanoma, 27/3143 (0.9%) prostate, and 15/8116 (0.1%) NSCLC cancers. MAPK pathway co-altered mutations were increased amongst *HRAS*-mutant prostate cancers (53.3%) compared to KRAS (17.6%) and NRAS (0%) tumors ($p<0.05$). PI3K pathway mutations were more prevalent in *HRAS*-mutant NSCLC cancers (53.3%) compared to *KRAS* (32.2%) and *NRAS* (21.4%) tumors ($p<0.05$). PI3K pathway mutations were also enriched in salivary tumors (63.2%). Multiple gene-level mutation differences were found within the MAPK pathway. *NF1* was the predominant mutation in *HRAS*-mutant melanoma (41%), compared to 20% and 8% of KRAS- and NRAS-mutant melanomas, respectively ($p<0.05$). In addition, class III *BRAF* mutations were found exclusively in *HRAS*-mutant tumors (salivary and bladder:5%, melanoma:9%) ($p<0.05$ vs *KRAS*- and *NRAS*-mutant tumors). Class II BRAF mutations were also enriched in *HRAS*-mutant tumors (bladder:5% and melanoma:9%) but only significantly different in melanoma (KRAS:0% and NRAS:0%, $p<0.05$). Thyroid cancer was the predominant cancer type carrying a co-altering *EIF1AX* mutation, although frequency did not differ amongst RAS-mutants (17% HRAS; 12% KRAS and 21% NRAS; $p=0.99$).

Conclusion: HRAS-mutant tumors demonstrated lineage-dependent increased cooperative events within the MAPK and PI3K pathway. Compared to *KRAS*- and *NRAS*-mutant tumors, *NF1* and class II/class III *BRAF* mutations were found at higher frequencies in *HRAS*-mutant melanoma and bladder cancers. The differences observed predict for distinct lineage and genotype-dependent responses to FTI treatment.

Inhibition of RAS signaling and tumorigenesis through targeting novel vulnerabilities

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RAS GTPases are important mediators of oncogenesis with nearly 30% of human tumors harboring mutant RAS proteins. However, pharmacological inhibition of RAS has proven challenging. We have employed Monobody technology to discover novel vulnerabilities in RAS that can be exploited to inhibit RAS signaling and tumorigenesis. Monobodies are single-domain synthetic binding proteins that achieve levels of affinity and selectivity similar to antibodies. In contrast to antibodies, Monobodies are fully functional in the reducing environment of the cytoplasm and thus are particularly suitable as genetically encoded "tool biologics". We previously developed the NS1 Monobody that inhibited RAS by targeting the α4-α5 allosteric lobe to prevent RAS self-association and nanoclustering, and NS1 has become a widely used tool in the RAS research community. Following on this success, we sought to identify additional vulnerabilities in RAS. Based on our discovery that nucleotide-free RAS (apoRAS) inhibits PIK3C2B function, we assessed the feasibility of selectively targeting this state of RAS as an approach to inhibit RAS. Here, we have developed several Monobodies and extensively characterized one of them, R15. Although NS1 was agnostic to the nucleotide state of RAS, R15 bound exclusively to the apo state of all three RAS isoforms but did not interact with nucleotide-loaded RAS. When expressed in cells, R15 selectively inhibited the signaling and transforming activity of RAS mutants with elevated intrinsic nucleotide exchange rates (i.e., "fast exchange mutants"), such as G13D and Q61L. Surprisingly, R15 bound and inhibited RAS(G12D) mutants which are not reported to be fast exchange. Biochemical studies demonstrated that RAS captured with R15 from cell lysates was indeed nucleotide free, suggesting that R15 traps apoRAS and prevents nucleotide reloading. Finally, intracellularly expressed R15 selectively inhibited the tumor forming capacity of human cell lines driven by fast exchange RAS mutants but not RAS(G12V) in mouse xenografts. Thus, in contrast to conventional wisdom, our approach has established a new opportunity to selectively inhibit certain RAS mutants by targeting the apo state of RAS with drug-like molecules.

Using Conformation-Selective RAF and MEK Inhibitors to Overcome Adaptive Drug Resistance and Retain Therapeutic Index in BRAF(V600E)-Driven Tumors

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BRAF kinase, a critical node in the RAS/MAPK signaling pathway, requires dimerization for its catalytic activity, whereas the oncoprotein BRAF(V600E) can be catalytically active as a monomer. Current clinical RAF inhibitors selectively bind and inhibit monomeric over dimeric BRAF and are now standard practice in combination with MEK inhibitors for the treatment of BRAF(V600E) tumors. However adaptive resistance due to RAF dimerization limits their effectiveness. Recently, RAF inhibitors equipotent against monomeric and dimeric RAF have been developed, but they are predicted to have lower therapeutic index due to inhibition of dimeric wild-type BRAF in normal tissue. Here we identify and characterize a third class of RAF inhibitors that preferentially binds and inhibits dimeric over monomeric RAF. Using Molecular Dynamics simulations, we observed distinct dynamic features of RAF inhibitors bound to monomeric and dimeric BRAF, thus providing a structural explanation for the selectivity of these drugs. Biochemical analysis using RAF dimer-selective inhibitors revealed that the two forms of dimeric and monomeric BRAF(V600E) differ in their conformation in their active site and their strength of interaction with MEK. To maximally inhibit BRAF(V600E) signaling in tumors while retaining a broad therapeutic index, we assessed the triple combination of RAF monomer- plus RAF dimer-selective inhibitor plus a MEK inhibitor that potently disrupts the BRAF-MEK complex. The triple combination potently suppressed tumor growth in multiple colorectal and melanoma BRAF(V600E) cell line and *in vivo* models that were resistant to the current clinical RAF and MEK inhibitor combination. Strikingly, while the double combination treatment with the RAF dimer-selective inhibitor and MEK inhibitor was associated with a significant gradual weight loss *in vivo*, the triple combination showed no weight loss or other apparent toxicities, indicating a higher therapeutic index. Finally, off-label use of the triple combination in a patient with a stage IV BRAF(V600E) colorectal cancer that progressed on standard therapies achieved durable tumor control with minimal toxicities. Thus, a rationally designed combinatorial approach of conformation-selective RAF and MEK inhibitors may be a highly effective and well tolerated therapeutic strategy for patients with BRAF(V600E) tumors.

Data science and informatics to enable RAS-related cancer research through the NIH *All of Us* Research Program and NCI Cancer Research Data Commons

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The NIH *All of Us* (AoU) Research Program is an initiative to collect broad biomedical and real-world data from diverse participants nationwide in order to accelerate precision medicine research. The NCI Cancer Research Data Commons (CRDC) is a cloud-based data ecosystem focused on catalyzing cancer research in particular. We built a biomedical informatics pipeline leveraging both resources in order to identify possible applications in RAS precision medicine cancer research.

We used the most recent (December 2020) release of the AoU dataset, with the cancer cohort defined by relevant ICD9 or ICD10 codes. A precision medicine drug was defined as any medication listed in the Food and Drug Administration's (FDA) Table of Pharmacogenomic Biomarkers in Drug Labeling. We used a cloud-based Jupyter Notebook (python 3.7, R 4.0.3) in the 1) NCI CRDC to create all cancer definitions and mapping files, and 2) AoU Researcher Workbench to perform all data extraction, integration, and analysis.

The informatics pipeline identified 26,038 cancer patients with 14,195,902 condition datapoints and 22,607 coded concepts for a variety of conditions and comorbidities, with 1,153,117 cancer condition datapoints and 1,489 cancer-related diagnosis codes. Further, there were 752 patients who received drugs with RAS biomarker information found in FDA drug labeling, with the three most common drugs being ramucirumab (645 patients), cetuximab (53 patients), and trametinib (22 patients). Key data research initiatives and resources like the NIH *All of Us* program and NCI CRDC can play important roles in future RAS-related cancer research.

Proteolytic RAS cleavage leads to tumor regression in patient-derived pancreatic cancer xenografts

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Effective RAS inhibition and treatment of pancreatic ductal adenocarcinoma (PDAC) represent major unmet medical needs in oncology. RRSP-DT_B is a novel engineered anticancer biologic that enters cells and cleaves the Switch I of all RAS isoforms. RRSP-DT_B was recently shown to reduce breast and colon tumors in xenograft studies. Here, we investigate the anticancer activity of RRSP-DT_B in *KRAS*-mutant PDAC cell lines and patient-derived xenografts (PDXs). We first demonstrate RRSP-DT_B effectively engages RAS and impacts downstream ERK signaling in multiple *KRAS*-mutant PDAC cell lines. Ras was cleaved in cells with IC_{50} ranging from 10-280 picomolar and cell proliferation was inhibited at 63-181 picomolar. A modified RRSP-DT_B that lacks catalytic activity was ineffective, demonstrating loss of proliferation is due to protease activity. We next tested RRSP-DT_B by administration to NSG mice bearing *KRAS*-mutant PDAC PDXs. Treatment led to ≥95% tumor regression after 29 days. Residual tumors exhibited disrupted tissue architecture, increased fibrosis and fewer proliferating cancer cells compared to controls. Levels of phospho-ERK were also significantly lower, indicating *in vivo* target engagement. Importantly, tumors that started to regrow in the absence of RRSP-DT_B shrank when treatment resumed, indicating resistance to RRSP-DT_B had not developed. A pharmacokinetic (PK) study showed RRSP-DT_B is active in sera of immunocompetent mice for at least one hour, but absent after 16 hours, justifying use of daily dosing. Overall, we report that RRSP-DT_B strongly regresses hard-to-treat *KRAS*-mutant PDX models of pancreatic cancer, warranting further development of this pan-RAS biologic for the management of RAS-addicted tumors.

Combined Inhibition of SHP2 and CXCR1/2 Promotes Anti-Tumor T Cell Response in NSCLC

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Clinical trials of SHP2 inhibitors (SHP2i) alone and in various combinations are ongoing for multiple tumors with over-activation of the RAS/ERK pathway. SHP2 plays critical roles in normal cell signaling; hence, SHP2is could influence the tumor microenvironment. We found that SHP2i treatment depleted alveolar and M2-like macrophages and promoted B and T lymphocyte infiltration in *Kras*- and *Egfr*-mutant non-small cell lung cancer (NSCLC). However, treatment also increased intratumor gMDSCs via tumor-intrinsic, NF-kB-dependent production of CXCR2 ligands. Other RAS/ERK pathway inhibitors also induced CXCR2 ligands and gMDSC influx in mice, and CXCR2 ligands were induced in tumors from patients on KRAS^{G12C}-inhibitor trials. Combined SHP2(SHP099)/CXCR1/2(SX682) inhibition depleted a specific cluster of *S100a8/9*^{high} gMDSCs, generated *Klrg1*+ CD8+ effector T cells with a strong cytotoxic phenotype but expressing the checkpoint receptor NKG2A, and enhanced survival in *Kras*- and *Egfr*-mutant models. Our results argue for testing RAS/ERK pathway /CXCR1/2/NKG2A inhibitor combinations in NSCLC patients.

Growth of Tumor with Mutated-KRAS Related Signals are inhibited by a dual VDAC1 and KDELR1 regulator

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It has been observed that several mutations of KRAS are present in 30% of all cancers. Additional mutations or the activation of genes associated with mutated (mt) KRAS, such as BRAF and AKT are also frequently observed in cancer. AMG510 targeting a cysteine residue of KRAS(G12C) (observed in 3% of cancers) using covalent inhibitors, however, other KRAS mutations remain undruggable. In this study, we developed the screening system of low molecular weight compounds from natural products targeting mtKRAS(G13D) related signals using human colorectal cancer (CRC) HCT116-drived, mtKRAS-disrupted (HKe3) spheroids exogenously expressing wild-type (wt) KRAS or mtKRAS. We found several compounds that selectively suppress HKe-mtKRAS spheroids. We selected the lead compound, STAR2, from an indole derivative for suppressing several cancer spheroids growth in vitro and HCT116 xenograft growth in vivo. Furthermore, we found Voltage-dependent anion channel (VDAC) 1 and KDEL receptor (KDELR)1 as STAR2 targets using pull down assays and fragment molecular orbital methods. Notably STAR2 inhibits several interactors binding through the N terminal domain of VDAC1 and/or the K-D-E-L binding motif of KDELR1. Furthermore, STAR2 suppress tumor-specific glucose metabolism (Warburg effect) through the suppression of the reactive oxygen species and subsequent HIF-1 transcription. These results together suggest that STAR2 is a low-toxic compound that specifically suppresses novel KRAS-related pathways.

KRAS A146 mutations are associated with distinct clinical behavior in patients with colorectal liver metastases

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Introduction: Somatic *KRAS* mutations occur in approximately half of the patients with metastatic colorectal cancer **(mCRC).** Biological tumor characteristics differ based on the *KRAS* mutation variant. *KRAS* mutations are known to influence patient prognosis and are used as predictive biomarker for treatment decisions. This study examined clinical features of mCRC patients with a somatic mutation in *KRAS* G12, G13, Q61, K117 or A146.

Methods: 419 CRC patients with initially unresectable liver-limited metastases, who participated in a multicenter prospective trial, were evaluated for tumor tissue *KRAS* mutation status. For the subgroup of patients who carried a *KRAS* mutation and were treated with bevacizumab and doublet or triplet chemotherapy (N=156), pretreatment circulating tumor DNA (ctDNA) levels were analyzed and total tumor volume (TTV) was quantified on the pretreatment CT images.

Results: Most patients carried a *KRAS* G12 mutation (N=112), followed by mutations in G13 (N=15), A146 (N=12), Q61 (N=9) and K117 (N=5). High plasma ctDNA levels were observed for patients carrying a *KRAS* A146 mutation *versus* those with a *KRAS* G12 mutation, with median mutant allele frequencies of 48% *versus* 19%, respectively. Radiological TTV revealed this difference to be associated with a higher tumor load in patients harboring a *KRAS* A146 mutation (median TTV 672cm^3 (A146) *versus* 74cm^3 (G12), p=0.036). Moreover, *KRAS* A146 mutation carriers showed inferior overall survival compared to patients with mutations in *KRAS* G12 (median 10.7 *versus* 26.4 months; HR=5.3; p=0.0045).

Conclusion: mCRC patients with a *KRAS* A146 mutation represent a distinct molecular subgroup of patients with higher tumor burden and worse clinical outcomes, who might benefit from more intensive treatments. These results highlight the importance of testing CRC for all *KRAS* mutations in routine clinical care.

Vertical Inhibition of the RAF-MEK-ERK Cascade Induces Myogenic Differentiation, Apoptosis and Tumor Regression in H/NRAS Q61X-mutant Rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common sarcoma in children with poor prognosis at advanced stage. Even when cured, children face profound lifelong health complications due to the cytotoxic and mutagenic nature of the current treatments. Identification of less toxic and more effective therapies is needed. Sequencing of fusion-negative rhabdomyosarcoma (FN-RMS) has identified high incidence of *RAS* mutations, with *NRAS* Q61X reported most frequently, presenting opportunity for design of targeted therapy strategies. By siRNA-mediated knock down of individual RAS isoforms we showed that H/NRAS Q61X-mutant FN-RMS cell lines exhibited oncogenic RAS dependency, and that knockdown of H/NRAS Q61X inhibited the ERK MAPK, but not PI3K-AKT pathway and phenocopied ERK inhibitor treatment by inducing myogenic differentiation. Further, as compared to RAS-wild type, H/NRAS-Q61X RMS cells exhibited preferential sensitivity to MEK/ERK, but not PI3K/AKT inhibitors. RNAseq analysis revealed significant overlap of genes signatures associated with NRAS-Q61H knockdown and ERK inhibition, indicating a central role of ERK in mediating H/NRAS Q61X-dependency in RMS. However, *in vivo* evaluation of the MEK inhibitor trametinib demonstrated poor response, which correlated with inefficient ERK inhibition in pharmacodynamic assays. CRISPR screen pointed to vertical inhibition of the RAF-MEK-ERK cascade by co-suppression of MEK and either CRAF or ERK. Concurrent CRAF suppression and MEK/ERK inhibition, or concurrent pan-RAF and MEK/ERK inhibition (pan-RAFi + MEKi/ERKi), or concurrent MEK and ERK inhibition (MEKi + ERKi) all blocked ERK activity and induced myogenic differentiation and cell death. *In vivo* evaluation of low dose pan-RAFi + ERKi or MEKi + ERKi potently suppressed H/NRAS Q61X RMS tumor growth, with pan-RAFi + ERKi being more effective and better tolerated, suggesting wider therapeutic window. Our results support the clinical translation of pan-RAFi + MEKi/ERKi for H/NRAS Q61X FN-RMS.

Signature-driven repurposing of FLT3 inhibitors for combination with MEK1/2 and KRASG12C targeted therapies

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The effect of single targeted agents in cancer is often compromised by intrinsic or adaptive resistance mechanisms, favoring the idea that combinatorial strategies are key to circumvent this clinical problem. For mutant KRAS-driven cancers, the development of such combinatorial strategies, including those based on KRASG12C inhibitors, has relied on the knowledge of KRAS effectors biology and/or information derived from mechanisms of resistance to MEK1/2 inhibitors. However, whether the distal gene expression output elicited by oncogenic KRAS can be interrogated for the prediction of the potential efficacy of drugs entering combinatorial strategies remains to be tested. Here, integrating a drug repurposing approach and a pairwise drug screen, we identified the combination of MEK1/2 and FLT3 inhibitors as a therapeutic opportunity for mutant *KRAS* lung cancer both in vitro (2D and 3D) and in vivo models. Furthermore, we show that a KRASG12C inhibitor can replace the MEK1/2 inhibitor with similar activity in all experimental models tested. Lastly, transcriptomics- and proteomics-based mechanistic studies uncover MYC as a downstream target mediating the effect to both drug combinations.

Targeting the RNA Binding Protein Igf2bp1 with a small molecule inhibitor: A novel therapy that down-regulates Kras mRNA, protein, and down-stream signaling

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The IGF2BP protein family consists of RNA binding proteins (RBPs) that have important roles during development, helping stabilize, localize, and translationally regulate mRNAs in embryonic cells., after birth;IGF2BP1&3 expression is dramatically down-regulated, In many types of cancers, however, IGF2BP1&3 is expressed and has been correlated with many pro-oncogenic processes, including proliferation, apoptosis, resistance to drugs, tumor progression, and metastasis. IGF2BP1 binds a number of specific mRNAs .One such pro-oncogenic mRNA is Kras. We have seen that Inhibition of IGF2BP1 expression in mouse models inhibits tumor metastases.

In collaboration with the Israel National Center for Personalized Medicine (INCPM), We have performed a high-throughput Fluorescence polarization (FP) screen for small molecule inhibitors of IGF2BP1 RNA binding.

By comparing fluorescently-labeled fragments of Kras mRNA, we identified with the FP a 200-NT sequence in its 3'UTR that binds IGF2BP1. Using this fragment, we scanned over 100,000 compounds that would prevent IGF2BP1 binding. This highly robust assay has yielded approximately 7reproducible hits dose-dependent (IC50around the tens of uM) that pass quality control by mass spectrometer. This molecules did not have an effect on a control RBP (La protein) binding to its target.

Compound 7773 was validated with orthogonal assays. Such as electrophoretic mobility shift assay(EMSA) and Microscale thermophoresis (MST).And structural analysis of the binding of 7773 to Igf2bp1 with NMR analysis indicates that 7773 associates with the RNA binding KH domains, at the C-terminus of the protein.
Strikingly When incubated cells with are leading hit, the compound targets Igf2bp1 and causes a reduction in Kras mRNA and other RNA targets, reduces Kras protein and downstream signaling, and represses cell migration and growth in soft agar.

Targeting p130Cas- and Microtubule-dependent MYC Regulation Sensitizes Pancreatic Cancer to ERK MAPK Inhibition

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To identify therapeutic targets for *KRAS*-mutant pancreatic cancer, we conducted a druggable genome siRNA screen and determined that suppression of *BCAR1* sensitizes pancreatic cancer cells to ERK inhibition. Integrative analysis of genome-scale CRISPR-Cas9 screens also identified *BCAR1* as a top synthetic lethal interactor with mutant KRAS. *BCAR1* encodes the SRC substrate p130Cas. We determined that SRC inhibitor-mediated suppression of p130Cas phosphorylation impairs *MYC* transcription through a DOCK1-RAC1-β-catenin dependent mechanism. Additionally, genetic suppression of *TUBB3*, encoding the βIII-tubulin subunit of microtubules, or pharmacological inhibition of microtubule function, caused calpain-dependent cleavage of MYC protein and potently sensitized pancreatic cancer cells to ERK inhibition. Accordingly, the combination of a dual SRC/tubulin inhibitor with an ERK inhibitor cooperated to reduce MYC protein and to synergistically suppress the growth of *KRAS*-mutant pancreatic cancer. Thus, we demonstrate that mechanistically diverse combinations with ERK inhibition suppress MYC to impair pancreatic cancer proliferation.

LentiMutate: Lentiviral-driven discovery of drug-resistant mutations

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Mutation-based methods to identify drug resistant mutations provide mutational profiles for specific drugs that can steer clinical targeted therapy strategies. Lentiviral transduction creates multiple types of mutations at baseline due to the error-prone nature of the HIV-1 reverse transcriptase (RT). We optimized and leveraged this property to identify resistance mutations to targeted drugs, a technique we term “LentiMutate”. This technique correctly identified known clinically relevant resistance mutations to imatinib, but also enabled discovery of new resistance novel deletions in BCR-ABL. LentiMutate also identified novel resistance mutations in KRAS^{G12C} to the covalent inhibitors AMG 510 and MRTX853. LentiMutate is valuable for prospective identification of drug resistance mechanisms and classification of targeted inhibitors.

Investigating $BRAF^{V600E}$/$NRAS^{Q61K}$ melanoma undergoing $BRAF^{V600E}$-directed intervention: Vemurafenib enhances $NRAS^{Q61K}$-associated EMT gene expression, invasiveness, and metastasis *in vivo*

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BRAF inhibitor (BRAFi)-resistance compromises long term survivorship of malignant melanoma patients. Mutant $NRAS^{Q61K}$ upstream of constitutive activation of PI3K/AKT/mTOR signaling is a major mediator of BRAFi-resistance, and it has been shown before that BRAFi therapy can accelerate pre-existing RAS-mutant malignancies including NRAS-mutant leukemia in melanoma patients. Here, employing NanoString transcriptomic analysis of isogenic (A375-$BRAF^{V600E}$ versus A375-$BRAF^{V600E}$/$NRAS^{Q61K}$) melanoma cells we demonstrate that BRAFi treatment selectively targets $BRAF^{V600E}$/$NRAS^{Q61K}$ cells with induction of Epithelial to Mesenchymal Transition (EMT) gene expression, paradoxically promoting invasiveness and metastasis *in vivo*. Cancer progression nCounter™ pathway analysis identified 'EMT' and 'Proliferative Control' gene expression networks specific to $BRAF^{V600E}$/$NRAS^{Q61K}$ status. Strikingly, in contrast to BRAFi (vemurafenib, VEM)-induced antiproliferative and anti-invasive effects in $BRAF^{V600E}$ cells, VEM enhanced proliferation and invasiveness of $BRAF^{V600E}$/$NRAS^{Q61K}$ cells. RT^2Profiler PCR array analysis confirmed VEM-upregulation of genes promoting EMT and proliferation [*AKT1*, *MMP3*, *PDGFRB*, *RAC1*, *SPARC*, *ZEB1*, *ZEB2* ($\leq$ 350-fold; $p<0.05$)] detectable only in $BRAF^{V600E}$/$NRAS^{Q61K}$ cells, while causing the expected downregulation of EMT-driver genes [*CDH2*, *FN1*, *FOXC2*, *IGFBP4*, *MMP9*, *VIM*, *WNT5A* ($\leq$ 40-fold; $p<0.05$)] only in the $BRAF^{V600E}$ isogenic variant. Phenotypic transwell migration assays confirmed the seemingly opposing effects of VEM treatment on melanoma cell invasiveness [achieving blockade ($BRAF^{V600E}$) or enhancement ($BRAF^{V600E}$/$NRAS^{Q61K}$)]. In a bioluminescent SCID mouse metastasis model using A375-luc isogenic variants, VEM treatment (50 mg/kg; p.o., q.d.) enhanced lung tumor burden imposed by $BRAF^{V600E}$/$NRAS^{Q61K}$ cells, while blocking metastasis of $BRAF^{V600E}$ cells. Our data provide preclinical evidence that identifies a BRAFi-driven upregulation of EMT-related gene expression potentially enhancing invasiveness and metastasis in human $BRAF^{V600E}$/$NRAS^{Q61K}$ melanoma.

An In-Vitro Study of Aromatic Stacking of paclitaxel and Antidepressants.

Amina S. Woods

Drug–drug chemical interactions between two different aromatic compounds were studied by mass spectrometry. Specifically, we examined non-covalent complexes (NCX) between paclitaxel, a chemotherapeutic compound, and medications widely used in palliative care for depression, psychosis, and anxiety. It is unknown whether psychotropic medications directly interact with paclitaxel. A simple and rapid electrospray ionization mass spectrometry in vitro assay, which has been predictive in the case of neuropeptides, to measure the relative strength of non-covalent interactions. This chemical interaction is most likely due to the overlap of aromatic rings of p-orbitals between paclitaxel and five commonly used medications: diazepam, clonozepam, sertraline, fluoxetine, and haloperidol. Molecular modeling illustrates that differences in the stability of the NCXs are likely due to the distance between the aromatic rings present in both the paclitaxel and antidepressant medications

Preclinical therapeutic synergy of MEK1/2 and IGF1R inhibition in RAS-driven rhabdomyosarcoma

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Background: Several pediatric solid tumors, such as rhabdomyosarcoma (RMS) and neuroblastoma (NB) are driven by alterations in the RAS/MAP kinase pathway and are partially responsive to MEK inhibition. Over-expression of the IGF1R as well as its ligands has been observed in multiple malignancies, including pediatric sarcomas and NB. Preclinical and clinical studies have suggested that IGF1R is itself an important target in these diseases. Previous studies revealed preclinical efficacy of the MEK1/2 inhibitor, trametinib, and an inhibitor of IGF1R, BMS75807, in cell line xenograft models of RAS-mutated RMS; however, clinical translation of this combination was limited by toxicity. Here, we sought to identify a combination of a MEK1/2 inhibitor and IGF1R inhibitor that would be better tolerated.

Methods: We studied the combinatorial effects of the MEK1/2 inhibitor trametinib, and the IGF1R monoclonal antibody ganitumab using proliferation and apoptosis assays in a panel of RAS-mutated RMS cell lines. The molecular mechanism of the observed synergy was determined using conventional and capillary immunoassays. The efficacy and tolerability of the combination was assessed using a panel of RAS-mutated cell-line and patient-derived xenograft models.

Results: Treatment with trametinib and ganitumab resulted in synergistic cellular growth inhibition in all cell lines tested and significant inhibition of tumor growth in five out of six models of RMS. The combination did not induce body weight loss, thrombocytopenia, or neutropenia in tumor-bearing SCID beige mice. Mechanistically, ganitumab treatment prevented the AKT phosphorylation that is induced by MEK inhibition alone. The therapeutic response to the combination correlated with the degree to which intra-tumoral AKT phosphorylation was inhibited.

Conclusions: We demonstrate that combined trametinib and ganitumab treatment shows therapeutic synergy across a wide panel of RAS-mutated preclinical models. These data support testing this combination in a phase I/II clinical trial for pediatric patients with relapsed or refractory RAS-mutated solid tumors.

Dual inhibition of DNA-PKcs and mTOR by CC-115 potently inhibits human renal cell carcinoma cell growth

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CC-115 is a dual inhibitor of DNA-PKcs and mTOR, both are valuable therapeutic targets for renal cell carcinoma (RCC). Our results showed that CC-115 inhibited survival and proliferation of established RCC cell lines (786-O and A489) and primary human RCC cells. The dual inhibitor induced selective apoptosis activation in RCC cells, as compared to no cytotoxicity nor apoptotic effects toward normal renal epithelial cells. CC-115 inhibited DNAPKcs and mTORC1/2 activation in RCC cells. It was however ineffective in DNA-PKcs-mTOR double knockout (DKO) 786-O cells. CC-115 induced feedback autophagy activation in RCC cells. Autophagy inhibitors or Beclin-1/Light chain 3 (LC3) silencing potentiated CC-115-induced anti-RCC cell activity. Conversely, ectopic overexpression of Beclin-1 inhibited CC-115-induced cytotoxicity. At last CC-115 oral administration inhibited 786-O subcutaneous xenograft growth in nude mice. Taken together, dual inhibition of DNA-PKcs and mTOR by CC-115 potently inhibited RCC cell growth.

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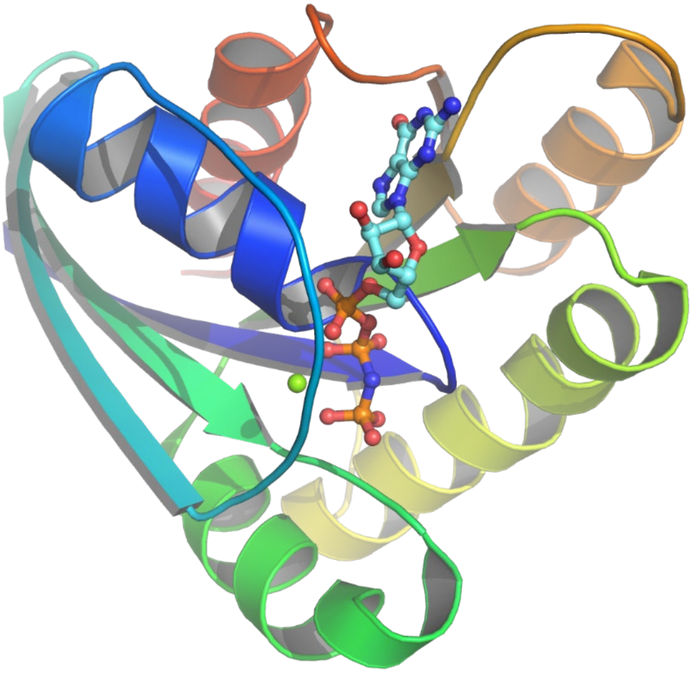

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Characterization of the KRAS Proteoform Landscape in Colorectal Cancer by Top-Down Proteomics

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The *RAS* GTPase family (*HRAS*, *NRAS*, and *KRAS)* has been identified as the most frequently mutated oncogenes in human cancer, with common mutations occurring at residues critical for GTPase activity (G12, G13, and Q61). KRAS4A and KRAS4B, produced by alternative splicing, bear numerous post-translational modifications (PTMs), which have been proposed to be critical for membrane association and localization, protein-protein interactions, and GTPase activity, along with potentially influencing the efficacy of anti-KRAS drugs.

Due to the high sequence identity between the RAS isoforms (up to 90%) and the loss of connectivity resulting from proteolytic digestion, "bottom-up" proteomic approaches (BU) have been unable to provide PTM stoichiometry and the precise molecular characterization needed to connect specific KRAS PTMs with a particular cancer-driving mutation. Our study employed a "top-down" (TD) proteomics approach to directly analyze the intact molecular forms, or proteoforms, of KRAS, thus revealing a more complete picture of the relationship between PTMs, KRAS isoforms, and mutational status. We have developed and optimized a method which couples KRAS immunoprecipitation with top-down mass spectrometry (IP-TDMS) and have applied it to investigate our hypothesis that KRAS4A and KRAS4B proteoforms will be mutation- or context-specific in a panel of 13 cell lines and 34 colorectal primary tumors. Our study has revealed previously unidentified KRAS proteoforms and provided unprecedented insight into the diverse KRAS proteoform landscape in colorectal cancer. We believe that the knowledge gained from our work will further our fundamental understanding of RAS biology as well as inform future anti-KRAS drug design.

High-resolution cryo-EM structure of full-length NF1

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Neurofibromin (NF1) is a GTPase-activating protein (GAP) and regulates the GTPase Ras, thus acting as a tumor suppressor. As a result, NF1 mutation and dysregulation is central to many diseases including neurofibromatosis, melanoma, leukemia and lung cancer. Understanding the structure-activity relationship of NF1 is crucial to developing effective therapeutic strategies to combat these diseases. While NF1 has been previously identified as a high-affinity dimer, high-resolution structural information of NF1 is currently limited to only the GAP related domain (GRD) and phospholipid binding domain (Sec-PH), encompassing around 20% of the protein. Thus, a more detailed and complete structure of the full-length protein is needed, to elucidate the chemical interactions that contribute to NF1 structural integrity and function, as this would provide mechanistic insights and serve as a guide to interpret the effects of many disease-associated mutations. In this presentation, we describe the cryo-EM structure of full-length NF1 and highlight important structural details.

Oncogenic BRAF mutations overcome negative regulation of RAF activity by ATP and promote dimerization to activate MAPK pathway signaling

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The RAS-RAF-MEK-ERK signaling axis is upregulated in many human cancers due to its central role in cell growth, differentiation and survival. Pathway activation depends on RAF dimerization, with oncogenic transformation frequently arising at the RAF node. BRAF mutations at V600 (Class I) release the kinase's activation segment rendering BRAF activity dimerization-independent. Yet, it remains unclear how a diverse set of dimerization-dependent BRAF mutations (Class II/III) activate the pathway. We recently reported a 2.9-Å-resolution crystal structure of human BRAF kinase domain in complex with MEK1 and the ATP analog AMP-PCP, revealing interactions between BRAF, MEK1 and ATP that induce an inactive, monomeric conformation of BRAF and explain how ATP breaks RAF dimers. Analysis of this structure reveals that all common oncogenic BRAF mutations, as well as oncogenic deletions to the MEK1 β3-αC loop, alter key interactions with ATP that break RAF dimers. We find ATP unable to break RAF dimers containing Class I, II or III mutations, confirming these mutants counteract the inhibitory effect of ATP binding by lowering the threshold for RAF dimerization and thus pathway activation. Our study establishes a framework for rationalizing oncogenic BRAF and MEK mutations and provides new avenues for improved and personalized RAF-inhibitor discovery.

Mutant RIT1 compromises mitotic fidelity through suppression of the spindle assembly checkpoint

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RIT1 is a Ras-related small guanosine triphosphatase (GTPase) that regulates cell survival and stress response and mutations in its gene have been identified as oncogenic drivers of lung adenocarcinoma and etiologic factors of Noonan syndrome. RIT1 has a unique set of effector proteins but shares activation of the MAPK pathway with other Ras GTPases. We have previously shown that RIT1 abundance and activity is regulated at the protein level through proteasomal degradation, a mechanism that is mediated by the adaptor protein LZTR1 and the E3 ubiquitin ligase Cullin 3. While RIT1's role in Noonan syndrome is likely mediated by the dysregulation of the MAPK pathway, a pathognomonic sign of the disorder, its role in normal cells and in malignancies is less clear. We have uncovered an unprecedended role for a Ras GTPase; the direct interaction of RIT1 with the spindle assembly checkpoint (SAC) to promote its inhibition. The SAC is an evolutionarily conserved safety mechanism that maintains genomic stability by delaying mitotic progression into anaphase until proper chromosome segregation is guaranteed. Disruptions to this safety mechanism lead to genomic instability and aneuploidy, which serve as the genetic cause of embryonic demise, congenital birth defects, intellectual disability, and cancer. We show that RIT1 is essential for timely progression through mitosis and proper chromosome segregation. Moreover, pathogenic levels of RIT1 silence the SAC, accelerate transit through mitosis, and promote chromosome segregation errors through direct association with SAC proteins MAD2 and p31^{comet}. We further demonstrate that suppression of the SAC is independent of RIT1's GTPase activity and requires the displacement of RIT1 from the plasma membrane during mitosis, a process that is regulated by CDK1-mediated phosphorylation of RIT1's HVR. While previous reports have implicated pathogenic Ras GTPase signaling in the dysregulation genomic stability, our results show a direct link between the SAC and a member of the Ras GTPase family, providing a novel example of the evolutionary adaptation of a signaling molecule for the regulation of a unique but critical cellular pathway.

Germline RASopathy mutations provide functional insights into the Raf cysteine-rich domain (CRD)

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Mutational activation of B-Raf is common in human cancer and RASopathy developmental syndromes. In contrast to oncogenic B-Raf mutations, which occur predominantly in the catalytic domain, RASopathy mutations also cluster around the cysteine-rich domain (CRD), a zinc finger-like domain in the B-Raf regulatory region. The CRD has been shown to play roles in Raf autoinhibition, phosphatidylserine (PS) binding and the Ras:Raf interaction, however the relative importance of these functions and CRD residues required for these activities are not well characterized. Through the in-depth analysis of a panel of RASopathy-associated B-Raf CRD mutations, we find that all of the mutations increase the biological activity of B-Raf by either relieving autoinhibition (Class A), relieving autoinhibition and enhancing PS affinity (Class B), or by enhancing PS affinity alone (Class C). We further show that each of these mutational classes have distinct effects on the subcellular localization, Ras binding properties, and biological output of B-Raf. Strikingly, when we compared the properties of the B-Raf CRD to that of C-Raf, we find that the B-Raf CRD is a stronger mediator of both autoinhibition and PS binding, and we identify specific residues that account for these differences. Moreover, due to the increased basal catalytic activity of B-Raf versus C-Raf, our studies indicate a more critical function for the CRD and autoinhibition in maintaining B-Raf in a non-signaling state, likely contributing to the high presence of B-Raf CRD mutations that disrupt this function in the RASopathies.

RRAS2 mutations act as fully autonomous cancer drivers in a variety of tumor types

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Hot-spot *RRAS2* gain-of-function (GOF) mutations have been identified at low frequency in both cancer and Noonan syndrome patients. The roles of this GTPase, however, remain poorly characterized *in vivo*. Here, we show that the endogenous wild-type and GOF mutant versions of R-RAS2 show a subcellular localization different from classical RAS GTPases. We also demonstrate using gene-editing techniques that *RRAS2* GOF mutations contribute to maintain both the fully transformed phenotype of cancer cells and optimal ERK and PI3K activation levels. These features are observed even in cancer cells bearing concurrent mutations in RAS signaling elements. Using a new tamoxifen-inducible knock-in mouse model, we have observed that the somatic expression of a GOF version of this GTPase (Q72L mutation) leads to the rapid emergence of a large variety of tumors such as T cell acute lymphoblastic leukemia, pre-pro B acute lymphoblastic leukemia, papillomas, fibrosarcomas and ovarian cystoadenomas. These tumors cannot be eliminated *in vivo* upon genetically depleting RalGDS or using MEK or PI3K inhibitors. By contrast, they can be ablated using mTORC inhibitors. Signaling studies utilizing cancer cells from these knock-in mice indicate that the endogenous R-RAS2^{Q72L} oncoprotein activates the mTORC pathway using convergent inputs from the MEK, PI3K and PDK pathways. These results indicate that *RRAS2* acts as a *bona fide* oncogene using overlapping, but not identical mechanisms to those elicited by RAS proteins. This information might be relevant for the understanding of the pathobiology and the therapeutic modeling of cancer and Noonan syndrome patients positive for *RRAS2* GOF mutations.

Pulsatile reactivation of MAPK signaling enables persistence of drug-adapted BRAF-mutant melanoma cells

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Targeted inhibition of oncogenic pathways can be highly effective in halting the rapid growth of tumors but often leads to the emergence of slowly dividing persister cells, which constitute a reservoir for selection of drug-resistant clones. In BRAF^{V600E} melanomas, RAF and MEK inhibitors efficiently block oncogenic signaling but persister cells emerge. Here, we show that persister cells escape drug-induced cell-cycle arrest via brief, sporadic ERK pulses generated by transmembrane receptor tyrosine kinases (RTKs) and growth factors operating in an autocrine/paracrine manner. Quantitative proteomics and computational modeling show that ERK pulsing is enabled by MAPK signaling rewiring: from an oncogenic BRAF^{V600E} monomer-driven configuration that is drug-sensitive to a receptor-driven configuration that involves Ras-GTP and RAF dimers and is highly resistant to RAF and MEK inhibitors. Altogether, this work shows that pulsatile MAPK activation by factors in the microenvironment generates a persistent population of melanoma cells that rewire MAPK signaling to sustain non-genetic drug resistance. A therapeutic implication is that residual disease in BRAF-mutant melanomas subjected to RAF/MEK inhibition is likely due to potent but transient MAPK reactivation in single cells. This suggests co-inhibition of the RTK- and Ras-driven signaling in BRAF-mutant melanoma to prevent non-genetic adaptation.

Targeting oncogenic RAS using next-generation modelling

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Oncogenic RAS mutations are frequent in cancer, yet RAS-targeted therapies are available only for a subgroup of RAS mutations. As a result, the inhibition of the kinases downstream of RAS has become a hot topic in drug development.
Clinically used RAF and MEK inhibitors are ineffective in RAS-mutant tumors and lead to reactivation of ERK signaling. A way to overcome resistance is the use of inhibitor combinations, but it is unclear how the best combinations can be chosen.
First, we analyzed which network effects can lead to drug resistance [Kholodenko et al., Cell Reports, 2021]. Next, we have focused on mechanisms of RAS-to-ERK pathway reactivation [Kholodenko, Cell Reports 2015]. We built a multi-scale mechanistic model to faithfully predict inhibitor responses at the network level [Rukhlenko et al., Cell Systems, 2018]. Counter-intuitively, our model suggested that resistance to RAF inhibitors in RAS mutant cells can be overcome by combining Type I½ and Type II RAF inhibitors.
To test model predictions, we measured responses of MEK/ERK signalling, proliferation and colony formation to different inhibitor types and their combinations in RAS-mutant cancer cell lines originating from acute myeloid leukaemia, melanoma, pancreatic, colorectal and non-small cell lung cancers. Our experiments corroborated model predictions, showing that two RAF inhibitors ineffective on their own can robustly suppress ERK pathway when used in combination and consist optimal two-drug combination for targeting oncogenic RAS.
Our results suggest a new principle of targeting the same kinase with two structurally different inhibitors that bind to different kinase conformations.

KRAS4A induces metastatic lung adenocarcinomas *in vivo* in the absence of the KRAS4B isoform

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In mammals, the *KRAS* locus encodes two protein isoforms, KRAS4A and KRAS4B, which differ only in their extreme C-terminus via alternative splicing of distinct fourth exons. Previous studies have shown that whereas KRAS expression is essential for mouse development, the KRAS4A isoform is expendable. In this study, we have generated a mouse strain that carries a point mutation in exon 4B that causes the selective degradation of the KRAS4B isoform while leaving KRAS4A expression unaffected. Mice selectively lacking KRAS4B developed to term but died perinatally due to hypertrabeculation of the ventricular wall, a defect reminiscent of that observed in midgestation embryos lacking the *Kras* locus. Mouse embryonic fibroblasts (MEFs) from *Kras4B*$^{-/-}$ embryos proliferated less than the corresponding *Kras* wild-type MEFs, due to limited expression of KRAS4A, a defect that can be compensated by ectopic expression of this isoform. Introduction of the same point mutation into a *Kras*^{FSFG12V} allele allowed expression of an endogenous KRAS4A^{G12V} oncogenic isoform in the absence of KRAS4B. Exposure of *Kras* $^{+/FSF4AG12V4B-}$ mice to Adeno-FLPo particles induced lung tumor formation with complete penetrance, albeit with increased latencies than control *Kras*$^{+/FSFG12V}$ animals. Moreover, a significant percentage of these mice developed proximal metastasis, a feature seldom observed in mice expressing both mutant isoforms. These results illustrate that expression of the KRAS4A^{G12V} mutant isoform is sufficient to induce lung tumors. Thus, raising the possibility that selective targeting of KRAS4B^{G12V} may not have significant therapeutic consequences.